THE MECHANISMS OF ANTIBIOTIC RESISTANCE IN CLINICAL ISOLATES OF *ACINETOBACTER* WITH SPECIAL REFERENCE TO CARBAPENEM RESISTANCE

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ABSTRACT

The most recent class of β-lactams, the carbapenems, have been an effective treatment for infections caused by multi-resistant strains of *Acinetobacter baumannii* however, there are now increasing reports of emerging carbapenem-resistant isolates world-wide, which severely threatens the successful treatment of patients infected by these bacteria. The mechanism of this resistance in *Acinetobacter* spp. has been attributed to a combination of reduced outer membrane permeability and loss of outer membrane proteins. More recently however, several reports have identified β-lactamases as a major mechanism. The main aim of this thesis was therefore to ascertain the importance of β-lactamase activity as a mechanism of carbapenem resistance in clinical isolates of *Acinetobacter* from several countries.

A total of 54 clinical isolates from Argentina, Turkey, Hong Kong, Spain and Singapore were investigated. The majority of isolates were identified as *A. baumannii*. Antimicrobial susceptibility testing detected resistance in all isolates to the majority of antimicrobials tested. A total of 69% of isolates demonstrated MIC values of imipenem above the recommended breakpoint value, of which the highest percentage resistance was observed in the isolates from Argentina and Singapore (89% and 82% respectively). A significantly lower level of resistance (38.9%) was observed to meropenem.

A modified microbiological assay detected imipenem inactivation in all imipenem-resistant isolates, of which 61% produced an unknown β–lactamase of pI 7.0 (main band). Inhibitor overlays of IEF gels demonstrated that this β–lactamase was a serine active-site enzyme that was able to bind imipenem. Kinetic studies with a crude β–lactamase extract of representative strain 790 failed to detect imipenem hydrolysis. A modified assay was subsequently employed to detect hydrolysis over a longer period of time. The rate of hydrolysis was calculated as 0.75 nmoles min^{-1} ml^{-1} of sample and the specific activity as 0.055 nmoles min^{-1} mg^{-1} of protein. Curing experiments with isolate 790 resulted in the loss of the β–lactamase of pI 7.0 and a subsequent reduction in the MIC value of imipenem to a susceptible level (16 mg/L to

0.5 mg/L). In addition, the modified microbiological assay detected an 11-fold decrease in the specific rate of imipenem hydrolysis with the cured strain (0.005 nmoles min^{-1} mg^{-1} of protein). These findings provided strong evidence that this β–lactamase was associated with plasmid-mediated carbapenemase activity. The enzyme was subsequently named ARI-2. Kinetic studies of ARI-2 following partial purification by anion exchange and gel filtration chromatography revealed that it was primarily a penicillinase. Slow hydrolysis of imipenem was detected, but not meropenem. Slow hydrolysis of oxacillin and cloxacillin was also detected, suggesting that ARI-2 belonged to molecular class D. This was further substantiated by inhibition of enzyme activity by sodium chloride. The M_r of ARI-2 was estimated as 35.5 kDa.

Extraction of plasmid DNA from isolate 790 revealed the presence of a plasmid of approximately 40 kb in the parent but not in the cured strain, which provided further evidence that the bla_{ARI-2} gene was plasmid-located. PCR analysis failed to reveal homology between bla_{ARI-2} and the class D ARI-1 β-lactamase. However, hybridization studies demonstrated some homology with this enzyme.

Analysis of ARI-2-producing isolates from Argentina, Turkey, Hong Kong and Spain by PFGE identified 5 main clusters. Four of these comprised strains from Argentina which suggested that horizontal transfer of the ARI-2 gene had occurred. Clonal spread was detected in the isolates from Turkey, which comprised the 5th cluster. The RFLP patterns of the single isolates from Spain and Hong Kong differed markedly from each other and from the main clusters.

Antimicrobial susceptibility studies of imipenem-resistant isolates detected variations in their sensitivities to 7 fluoroquinolones. Significant resistance was demonstrated to ciprofloxacin and norfloxacin however, the newer quinolones (sparfloxacin, trovafloxacin, grepafloxacin and moxifloxacin) were more active with significantly lower MIC_{50} and MIC_{90} values observed. A total of 62% of imipenem-resistant isolates from Argentina were resistant to all of the fluoroquinolones tested. A

serine-83 to leucine *gyr*A mutation was detected in all isolates with a ciprofloxacin MIC value of ≥ 2 mg/L. The majority of isolates with ciprofloxacin MIC values of ≥ 16 mg/L also contained a serine-80 to leucine *parC* mutation. However, this mutation was not present in 5 isolates with ciprofloxacin MIC values of 16 mg/L. A Glu-84 to Leu change was detected in one of these isolates, which suggested that different mutations, or indeed other mechanisms of quinolone resistance may be important in *Acinetobacter*.

A study of the efficacy of sulbactam was performed with carbapenem-sensitive clinical isolates from Scotland and with carbapenem-resistant isolates form Argentina, Spain and Hong Kong. The lowest MIC values were demonstrated with the carbapenem-sensitive isolates from Scotland (MIC range of 0.5 - 2.0 mg/L). The highest MIC values were observed with the isolates from Argentina (MIC range 2 - ≥16 mg/L). Sulbactam did not have a significant inhibitory effect against isolates with MIC values of ≥ 4 mg/L at concentrations above this level. However, a decrease in growth rate of an isolate with a MIC value of 0.5 mg/L was detected. The combined activity of imipenem and sulbactam was evaluated. Although bactericidal synergy was detected with one strain, this combination failed to produce a similar effect with 2 other strains, suggesting that the combined effect of imipenem and sulbactam cannot be predicted and is very much strain-dependent.

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

ACKNOWLEDGEMENTS

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Last but not least, I would like to convey my thanks to Gary for his endless encouragement, belief in me and, above all else, his patience. I will always be grateful.

This thesis is dedicated to the treasured memory of my uncle Jim who sadly died during the final stages of its completion.

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PUBLICATIONS AND PRESENTATIONS

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ABBREVIATIONS

Abs	absorbance
7-ACA	7-amino-cephalosporanic acid
Ala	Alanine
AMOX	Amoxycillin
6-APA	6-amino-penicillanic acid
APH(6)	aminoglycoside 6-phosphotransferase
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
bla	β-lactamase gene
bp	base pair
CAZ	Ceftazidime
CIP	Ciprofloxacin
CLD	Cephaloridine
CO-AMOX	Co-amoxiclavulanic acid
CTX	Cefotaxime
Cys	Cysteine
DHP-I	dehydropeptidase I
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
EDTA	ethylene diaminetetraacetate
FIC	fractional inhibitory concentration
F1-dUTP	fluorescein-11-dUTP
FPLC®	Fast Protein Liquid Chromatography
GEN	Gentamicin
Glu	Glutamic acid
Gly	Glycine
GREPA	Grepafloxacin
HCl	hydrochloric acid

His	Histidine
ID_{50}	concentration of inhibitor required to inhibit 50% of enzyme activity
IEF	isoelectric focusing
IMP	Imipenem
IS	insertion sequence
IST	IsoSensitest
ITU	intensive therapy unit
kb	kilobase
K_m	Michaelis constant
L	litre
LB	Luria-Bertani
Leu	Leucine
Lys	Lysine
M	molar
mA	milliamperes
MERO	Meropenem
µg	microgram
µl	microlitre
µM	micromolar
ml	millilitre
mM	millimolar
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration for 50% of strains
MIC_{90}	minimum inhibitory concentration for 90% of strains
$\Delta\varepsilon$	molar extinction coefficient of hydrolysis
MOXI	Moxifloxacin
ND	not detected
nm	nanometre
NORFLOX	Norfloxacin
NT	not tested
OD	optical density

OMP	outer membrane protein
PBP	penicillin-binding protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
pI	isoelectric point
Pro	Proline
PVDF	polyvinylidene difluoride
QRDR	quinolone resistance-determining region
RFLP	restriction fragment length polymorphism
RPHPLC	Reversed-Phase High-Performance Liquid Chromatography
rRNA	ribosomal ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	sodium chloride/EDTA
Ser	Serine
SPAR	Sparfloxacin
Spp.	species
TAE	tris acetate
TBE	tris-borate-EDTA
TE	tris/EDTA
TEMED	tetramethyl-ethylenediamine
TFA	trifluoroacetic acid
tris	tris(hydromethyl)methylamide
TROVA	Trovafloxacin
Tyr	Tyrosine
V	volt
Val	Valine
V_e	volume of enzyme
V_{max}	maximum rate of hydrolysis
V_r	volume of reaction
v/v	volume per volume
w/v	weight per volume

W	watt
λ	wavelength of light

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CHAPTER 1

INTRODUCTION

1.1 The post-antibiotic era?

In 1969, the US Surgeon General testifying to Congress stated rather ambitiously, "The time has come to close the book on infectious disease". As we enter a new century, many microbiologists believe that nothing is further from the truth, and that we are, in fact, on the verge of a medical disaster that will return us to the days before penicillin where minor infections may prove lethal because we do not have the antibiotic 'armoury' to deal with them (Kendell, 1994). This unthinkable scenario has been attributed mainly to the remarkable ability of bacteria to adapt and survive, no matter what antimicrobial bullets are fired at them. Clinicians are now all too frequently faced with treating patients infected by multi-resistant bacteria which are often susceptible to only one or two antibiotics at the most. This is an extremely worrying situation, considering there are now over 250 antibiotics available commercially. The market was crowded with many of these compounds in the 1980s, which many believe led to a degree of complacency on the part of the pharmaceutical industry, perhaps assuming the battle against bacterial infections had already been won.

Today, the heavy use of antibiotics in farm animals and the selective pressure exerted by over-use of many of these broad-spectrum drugs in hospitals, has ensured that bacteria continue to develop resistance at an alarming rate. Moreover, the organisms responsible for many hospital, or nosocomial infections, are evolving continuously. Bacteria such as *Acinetobacter* and *Stenotrophomonas* species were considered to be of relatively low virulence in humans. Now, they are accepted pathogens in infections of intensive therapy unit (ITU) patients. Scientists are now having to broaden their horizons beyond the development of antimicrobial analogues and are endeavouring to search for new therapeutic strategies.

1.2 An historical background to the antibiotic era

Although both natural and artificial substances have been used to treat infectious diseases throughout history, many of these treatments were purely topical (Selwyn, 1983). It was not until the twentieth century that agents with antimicrobial properties were studied for treating systemic disease.

Paul Ehrlich did much to bridge the gap between the dye industry and microbiology with his research on aniline dyes and arsenical compounds for the treatment of protozoan diseases. He based his experiments on the important and fundamental principle of 'selective toxicity' (Ehrlich *et al.*, 1913). Subsequently, the bacteriologist, Gerhard Domagk demonstrated that a dye, prontosil red, could be used to cure experimental mice infected with *Streptococcus pyogenes* (Domagk, 1935). It was noticed, however, that the antibacterial activity of this dye could not be demonstrated *in vitro*. A research group in France finally solved this mystery by demonstrating that the active agent, a sulphanilamide group, was enzymatically released from the dye complex within the body (Tréfouël *et al.*, 1935). By altering the nature of the substitution on the amino group of this moiety, chemists were able to produce pharmacokinetically improved compounds, resulting in synthetic sulphonamides which heralded the start of the chemotherapeutic era as we know it today.

1.2.1 The β-lactams- antibiotics from the soil

1.2.2 The penicillins

The discovery of the first of this important class of antibiotics, penicillin, by Alexander Fleming in 1929 is renowned, and is considered one of the most significant medical findings of our age (Fleming, 1929). As was the case with several of the naturally-occurring antibiotics, its discovery was due mainly to a chance observation.

Fleming noticed that a mould, *Penicillium notatum*, which had contaminated a culture plate of *Staphylococcus aureus*, inhibited growth of the bacteria. The crude material originally extracted from the mould was found to include a number of compounds duly named penicillins F, K, X and G. The latter, also known as benzylpenicillin, was the most active and was subsequently chosen for further development.

One of the first documented uses of penicillin as a treatment was in 1930 when a doctor who was a former pupil of Alexander Fleming, used a crude extract of *Penicillium* to successfully treat a case of ophthalmia neonatorum (reviewed by Wainwright and Swan, 1986). However, due to difficulties in production and purification, the clinical value of penicillin as an effective chemotherapeutic agent was not realised until the mid-1940s, mainly due to the pioneering work of Howard Florey and Ernst Chain in Oxford (Chain *et al,* 1940).

The isolation of the penicillin nucleus, 6-amino-penicillanic acid (6-APA) in 1959 was a major event in the history of the β-lactams as it allowed the addition of novel side-chain structures and thus paved the way for the production of many semi-synthetic penicillins (Rolinson, 1979). One of the main objectives of the work on 6-APA was to produce new penicillins which would be effective against the increasing number of penicillin-resistant staphylococci already emerging in hospitals (Barber and Whitehead, 1949). Indeed, these strains started to appear within a few years of the widespread use of penicillin G. The cause of this resistance was due to the production of an enzyme penicillinase (β-lactamase) which inactivated the antibiotic by hydrolysing the β-lactam ring (Kirby, 1944). By the time 6-APA had been isolated, the incidence of resistance was as high as 80% in many hospitals (Rolinson, 1979). To date, a vast number of penicillins have been developed in an attempt to counteract the ever-increasing problem of bacterial resistance.

1.2.3 The cephalosporins

The discovery of penicillin prompted many scientists to look for new antibiotic-producing organisms. In 1945, a bacteriologist, Giuseppi Brotzu, studied the microbial flora of seawater near a sewage outfall in Sardinia, and came across a strain of *Cephalosporium acremonium* which secreted a substance with activity against a number of Gram-positive and Gram-negative bacteria. Due to lack of facilities and expertise, he passed a culture of the mould to Florey's team in Oxford for further analysis. Their studies revealed a number of antibiotic substances. However, it was not until the mould was grown on a much larger scale that these were identified.

The first was named cephalosporin P because it demonstrated activity against certain Gram-positive bacteria (Burton and Abraham, 1951). Sufficient amounts of this could not be obtained for clinical trials. However, another antibiotic substance, cephalosporin N was discovered from the extraction procedure used to obtain cephalosporin P. This was active against both Gram-negative bacteria and penicillin-sensitive staphylococci, and was undoubtedly the same substance that Brotzu had originally found. It was then revealed that this antibiotic was inactivated by a penicillinase from *Bacillus cereus* (Newton and Abraham, 1954) and was subsequently renamed penicillin N. Because of the difficulties in successfully isolating it, this compound was never produced in quantity by the pharmaceutical industry, however, a small amount of another substance was found during an attempt to establish the molecular formula of penicillin N by chromatography.

This was named cephalosporin C, the first and original member of the class of antibiotics known as the cephalosporins (Newton and Abraham, 1955). Unlike penicillin N, cephalosporin C was relatively acid-stable and demonstrated a high resistance to inactivation by *B. cereus* and staphylococcal penicillinases. Although it was used in the treatment of urinary tract infections in children (Fleming, 1963), cephalosporin C did not play a major role in the treatment of infections caused by penicillinase-producing

staphylococci, as by this time the semi-synthetic penicillins were very much in the therapeutic spotlight.

The chemical structure of cephalosporin C was eventually confirmed by x-ray crystallography (Crowfoot and Maslen, 1961), which allowed the identification of two groups within the structure that could potentially be modified. As with the development of the penicillins, the nucleus of cephalosporin C, 7-amino-cephalosporanic acid (7-ACA), needed to be isolated if this was to prove successful. Although attempts were made to achieve this, it was not until the pharmaceutical industry became involved that an efficient chemical method of isolation was found which allowed 7-ACA to be prepared in large quantities (Morin *et al.*, 1962). The semi-synthetic cephalosporins had arrived.

The common feature of the β-lactams is the presence of a 4-membered β-lactam ring structure (Figure 1.1) which is essential for antimicrobial activity.

1.3 Structure of the penicillin nucleus

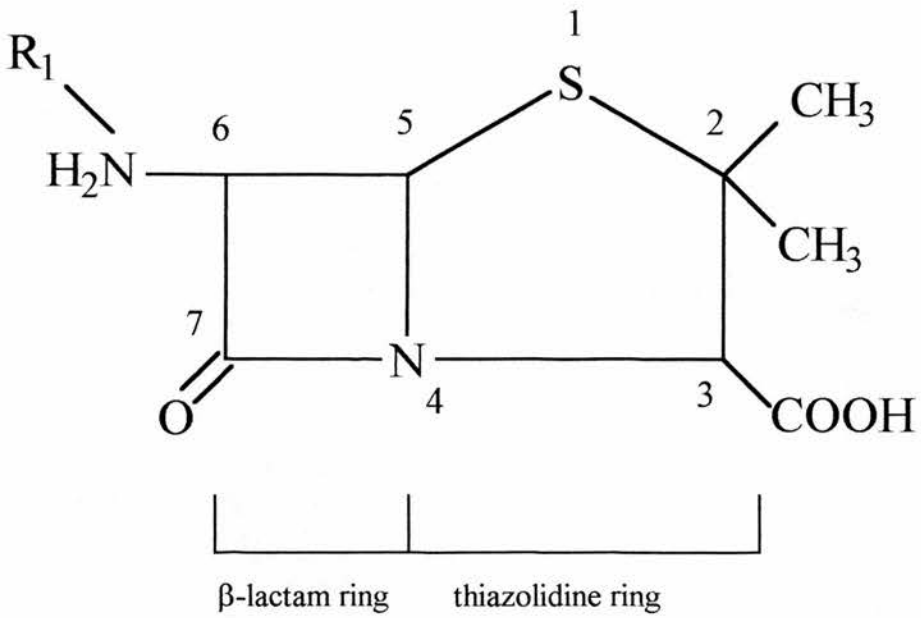
The penicillins, or penams, are characterised by a fused heterocyclic structure which consists of the β-lactam ring, a 5-membered sulphur-containing thiazolidine ring and a side chain (Figure 1.1). Manipulation of this side chain essentially alters the spectrum of activity and pharmacokinetics of the compound (Figure 1.2). Unlike the cephalosporins, the C_6 position of the molecule is the only site on the nucleus that can be substituted.

1.3.1 Classification of the penicillins

Benzylpenicillin, exhibits good activity against many Gram-positive cocci and Gram-positive bacilli however, it is susceptible to staphylococcal β-lactamases and is labile to gastric acid. The objective of early research following the isolation of 6-APA

was therefore to synthesise narrow-spectrum penicillins similar to benzylpenicillin but with better oral absorption qualities, and to produce compounds which were stable to staphylococcal β-lactamases and had activity against penicillin-resistant staphylococci (Figure 1.3).

Figure 1.1 Structure of 6-amino-penicillanic acid (6-APA)



The natural penicillins

Benzylpenicillin is still the choice drug for many Gram-positive infections. Phenoxymethylpenicillin (penicillin V) (Figure 1.2) is an oral derivative of penicillin which has similar antibacterial properties to those of benzylpenicillin but resists degradation by gastric acid.

Semi-synthetic penicillinase-resistant penicillins

These compounds have an acyl side-chain that sterically inhibits the action of penicillinase by preventing opening of the β-lactam ring. Methicillin was the first of this group to be developed (Figure 1.2). Others include oxacillin, nafcillin, cloxacillin and dicloxacillin (the latter two are for oral use). They have good activity against both *Staphylococcus* species and *Streptococcus* species, but no activity against Gram-negative bacteria. Although these antibiotics are often first choice therapy for many staphylococcal infections, the emergence of methicillin-resistant *S. aureus* in the early 1980s has curtailed their usefulness (Cookson and Phillips, 1990).

Extended-spectrum penicillins

Aminopenicillins

These were the first group of penicillins developed with activity against Gram-negative bacteria. Ampicillin is an α-amino derivative of benzylpenicillin (Figure 1.2). Amoxycillin is closely related to ampicillin in both chemical structure and antibacterial activity but has better absorption. Both initially had activity against many Gram-negative bacteria (except *Klebsiella* and *Pseudomonas* spp.), including *Escherichia coli*, *Proteus mirabilis*, *Salmonella* and *Shigella* spp., but resistance has limited their use against many of these strains.

Carboxypenicillins

Carbenicillin was developed by the substitution of the amino group of ampicillin for a carboxyl group (Figure 1.2). Subsequent substitutions on carbenicillin produced the

thienyl variant, ticarcillin. Both have improved activity against Gram-negative bacilli, including *P. aeruginosa*, which is intrinsically resistant to most of the β-lactams.

Ureidopenicillins

Examples of these ampicillin derivatives include mezlocillin and azlocillin (Figure 1.2). The major advantage of this group of penicillins is their enhanced activity against *P. aeruginosa*.

β-lactamase inhibitors

This strategy was approached to counteract resistance as a result of β-lactamase production. Beta-lactamase inhibitors have only weak antibacterial activity but increase the activity of certain β-lactams when they are combined with them (Maiti *et al.*, 1998).

Clavulanic acid

This is a bicyclic oxapenam (Figure 1.2) produced by *Streptococcus clavuligerus* (Brown *et al.*, 1976; Reading and Cole, 1977), and is a powerful inhibitor of most class A and some class D β-lactamases. Classes B and C are unaffected. Clavulanic acid is used in combination with amoxycillin or ticarcillin. Its efficacy has prompted the production of several semi-derivatives however, the original inhibitor remains the most effective (Maiti *et al.*, 1998).

Sulbactam

This penam sulphone (Figure 1.2) displays potent inhibitory activity against primarily class A enzymes, but also has a greater affinity for class C β-lactamases than clavulanic

acid (English *et al.*, 1978). It is commercially available in combination with ampicillin, or in some countries with cefoperazone.

Tazobactam

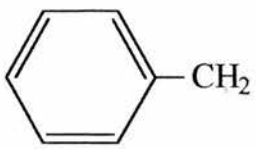
This inhibitor is a penam sulphone derivative (from modification of the 2-methyl groups of sulbactam) that has a broader spectrum of inhibitory activity than sulbactam and clavulanic acid, which includes most class A and some class D β-lactamases, and the AmpC enzymes of Enterobacteriaceae (Aronoff *et al.*, 1984; Maiti *et al.*, 1998).

BRL 42715

This belongs to a class of 6-(substituted methylene) penem inhibitors (Bennett *et al.*, 1991), and is a good inhibitor of a broad range of β-lactamases with a serine residue at their active-site (Farmer *et al.*, 1994). However, as a result of its chemical instability and short half life in humans, it is no longer produced for clinical use.

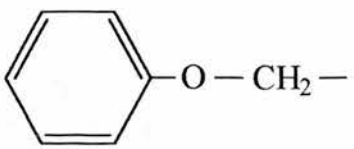
Figure 1.2 Chemical structures of the R1 penicillin side-chains

natural penicillins



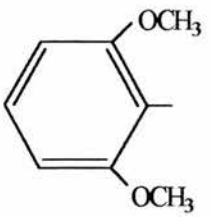
e.g. Penicillin G

orally absorbed penicillins



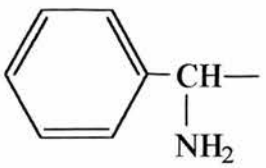
e.g. Penicillin V

semi-synthetic penicillinase-stable penicillins



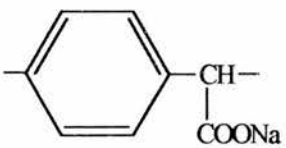
e.g. Methicillin

aminopenicillins



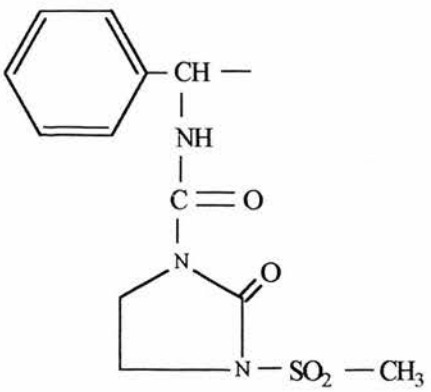
e.g. Ampicillin

carboxypenicillins



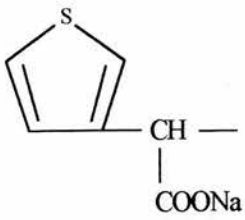
e.g. Carbenicillin

acylureidopenicillins



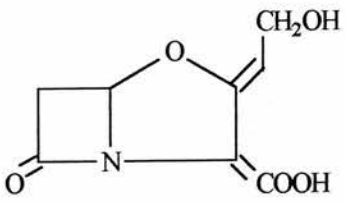
e.g. Mezlocillin

β-lactamase-stable penicillins

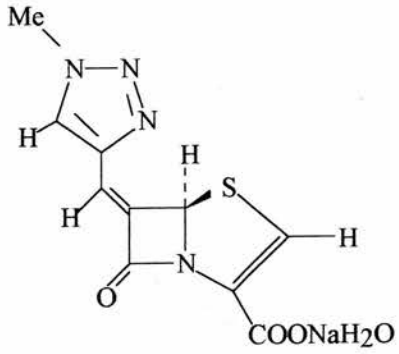


e.g. Temocillin

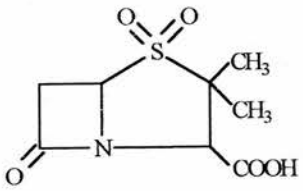
β-lactamase inhibitors



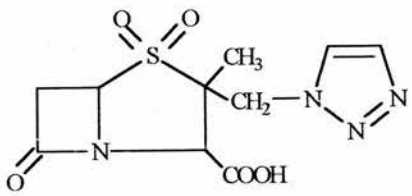
Clavulanic acid



BRL 42715



Sulbactam



Tazobactam

Figure 1.3 Classification of the penicillins

NATURAL PENICILLINS:

Benzylpenicillin

Long acting parenteral forms
Benethamine penicillin
Procaine penicillin
Clemizole penicillin

ORALLY ABSORBED PENICILLINS SIMILAR TO BENZYLPENICILLIN

Phenoxypenicillins

Phenoxymethyl penicillin (pen V)

Phenethicillin

Phenoxypropylpenicillin

SEMI-SYNTHETIC STAPHYLOCOCCAL-STABLE PENICILLINS

Methicillin
Nafcillin

Isoxazolyl penicillins
Cloxacillin
Flucloxacillin
Dicloxacillin
Oxacillin

EXTENDED-SPECTRUM PENICILLINS

Aminopenicillins
Ampicillin
Amoxycillin
Cyclacillin
Mecillinam

Ampicillin condensates
Hetacillin
Metampicillin

Ampicillin esters
Bacampicillin
Lenampicillin
Pivampicillin
Talampicillin

mecillinam ester: Pivmecillinam

PENICILLINS ACTIVE AGAINST *Pseudomonas aeruginosa*

Carboxypenicillins
Carbenicillin
Ticarcillin

Carbenicillin esters
Carfecillin
Carindacillin

Acylureidopenicillins
Azlocillin
Mezlocillin
Piperacillin

β-LACTAMASE-RESISTANT PENICILLINS

Temocillin (6 α-methoxy derivative of ticarcillin)

PENICILLIN PLUS β-LACTAMASE INHIBITORS

Amoxycillin-Clavulanic acid
Ticarcillin-Clavulanic acid
Ampicillin-Sulbactam
Piperacillin-Tazobactam

Adapted from Wright, 1999; Sutherland, 1997

1.4 Structure of the Cephalosporins

All the cephalosporins have a 6-membered dihydrothiazine ring fused to the β-lactam ring (Figure 1.4). The extra carbon of the ring carries an additional side chain, the nature of which often affects the pharmacokinetic behaviour of the molecule, and in some cases, the degree of toxicity. A major advantage of the cephalosporins over the penicillins is that there are two positions (R_1 and R_2) on the molecule that can be modified (Figures 1.4 and 1.5).

Figure 1.4 Structure of 7-aminocephalosporanic acid (7-ACA)

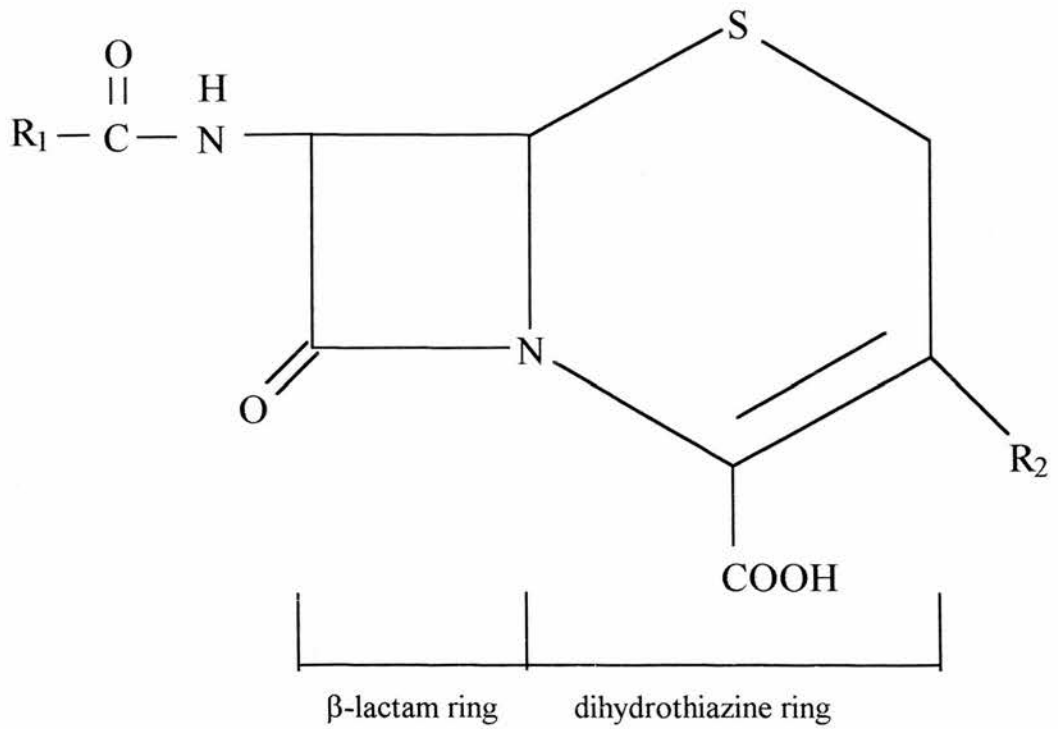
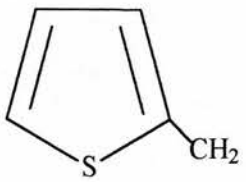
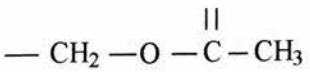


Figure 1.5. Chemical structures of the R1 and R2 cephalosporin side-chains

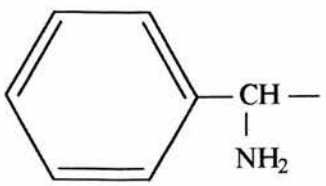
R1 **R2**

Group 1 - e.g. Cephalothin

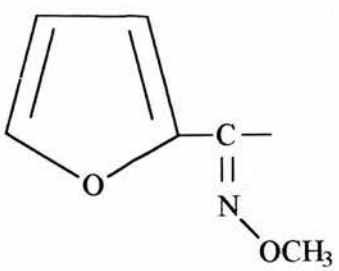


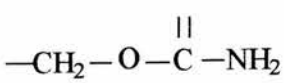


Group 2 - e.g. Cephalexin

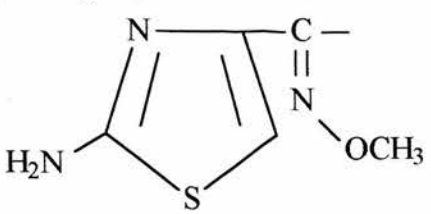


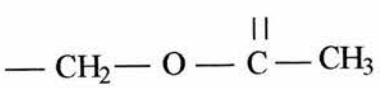
Group 3 - e.g. Cefuroxime





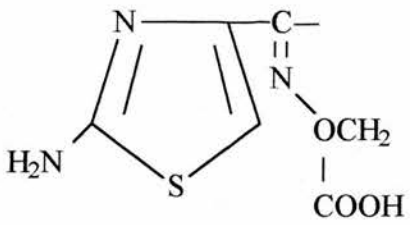
Group 4 - e.g. Cefotaxime

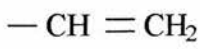




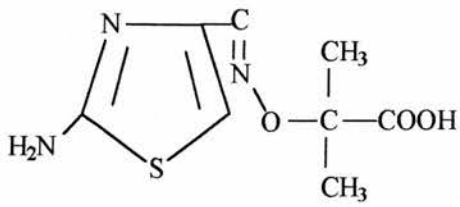
R1 **R2**

Group 5 - e.g. Cefixime

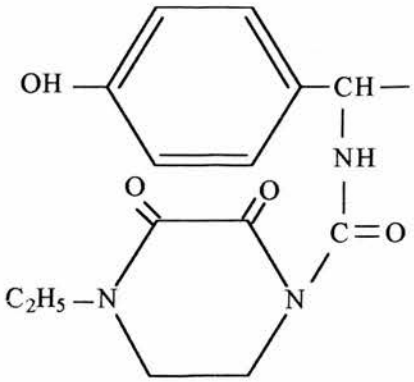


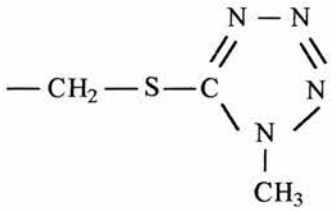


Group 6 - Broad-spectrum e.g. Ceftazidime

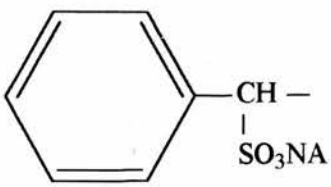


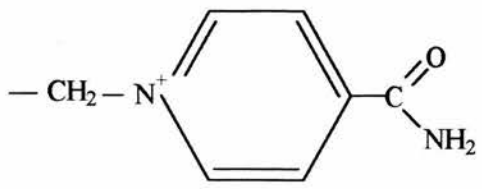
Group 6 - Medium-spectrum e.g. Cefoperazone





Group 6 - Narrow-spectrum e.g. Cefsulodin





1.4.1 Classification of the cephalosporins

The groups listed below differentiate these compounds based on the route of administration, their degree of antimicrobial activity and stability to bacterial β-lactamases. Members of each group are listed in figure 1.6.

Group 1

These parental compounds were developed in response to a need for more effective antibiotics against staphylococci. They have moderate antimicrobial activity and have relative stability to staphylococcal β-lactamase. However, they are hydrolysed by many of the Enterobacteriaceae. The first compounds to be developed in this group were cephalothin and cephaloridine.

Group 2

Compounds within this group are administered orally and demonstrate moderate resistance to some of the enterobacterial β-lactamases. The first of these compounds to be developed was cephalexin which has a methyl group at C_3 (Figure 1.5). This was followed by cefaclor, which has a chlorine in place of the methyl group, and cephradine, which has the phenyl group of cephalexin replaced by a cyclohexadiene group.

Group 3

These are all parental compounds and include the related group the cephamycins which share the common feature of a methoxy group attached to the 7-α-position of the β-lactam ring conferring stability to the structure. This group displays moderate antimicrobial activity and includes activity against the anaerobe, *Bacteroides fragilis*.

Group 4

This group includes parenteral compounds with potent antimicrobial activity and resistance to a wide range of β-lactamases. They are characterised by the presence of an N-acyl side-chain containing a 2-aminothiazole group (Figure 1.5).

Group 5

The compounds within this group originate from an attempt to improve the intrinsic activity of the previous oral cephalosporins. Although they have improved activity against many of the enterobacteria, their spectrum against the Gram-positives is not as good.

Group 6

These parenteral compounds are characterised by their activity against *P. aeruginosa* and resistance to a wide range of β-lactamases.

Group 7

This group includes the parenteral compounds cefepime and cefpirome which have enhanced β-lactamase stability, potent activity against enterobacteria and enhanced activity against staphylococci.

Groups 1 and 2 are often referred to as the 'first' generation cephalosporins, group 3 the second, groups 4, 5 and 6 the third, and group 7 the fourth generation.

Figure 1.6 Classification of the cephalosporins

GROUP 1 - Parenteral, relative stability to staphylococcal penicillinase

Cephalothin	Cephapirin	Cefonicid
Cephaloridine	Ceftezole	Cefazedone
Cephazolin	Ceforanide	Cephacetrile

GROUP 2 - Oral

Cephalexin	Cephaloglycin	Cefatrizine
Cephradine	Loracarbef (cephamycin)	Cefroxadine
Cephaclor	Cefadroxil	Cefporzil

GROUP 3 - Parenteral, improved β-lactamase stability

Cefuroxime	Cephamandole	Cefmetazole*	
Cefotiam	Cefoxitin*	Cefbuperazone*	*Cephamycins
Cefonicid	Cefotetan*		

GROUP 4 - Parenteral, improved intrinsic activity and β-lactamase stability

Cefotaxime	Ceftriaxone	Cefodizime
Ceftizoxime	Cefmenoxime	Latamoxef (Oxacephem)

GROUP 5 - Oral, improved β-lactamase stability

Cefdinir	Cefprozil	Cefetamet	Ceftibuten
Cefixime	Cefteram	Cefpodoxime	

GROUP 6 - Parenteral, activity against *P. aeruginosa*

Broad spectrum - Ceftazidime

Medium spectrum - Cefoperazone
Cefpimazole
Cefpiramide

Narrow spectrum - Cefsulodin

GROUP 7 - Parenteral, enhanced β-lactamase stability, potent activity against enterobacteria

cefipime cefpirome

Adapted from Wise, 1997

1.5 Other β-lactams

There are a vast number of antimicrobial agents that are related to the penicillins and cephalosporins (Figure 1.7). Those with therapeutic use are described below.

1.5.1 Oxacephems, cephamycins and carbacephems

These compounds are all closely related to the cephalosporins. The sulphur of the oxacephems and the carbacephems are substituted by an oxygen and a carbon respectively. The cephamycins have a α-methoxy group at position 7 (Figure 1.7).

1.5.2 Monobactams

This group of natural monocyclic compounds (which also includes the nocardicins) are produced by bacteria. They frequently have a α-methoxy group attached to the β-lactam ring, a feature shared by the cephamycins. Aztreonam was the first of these compounds to be clinically used and is active against a wide range of aerobic Gram-negative bacteria.

1.5.3 Penems

These synthetic compounds differ from the penams by the presence of a double bond between C_2 and C_3.

1.5.4 Carbapenams

The carbapenams differ from the penams in the substitution of a CH_2 group for sulphur in the five-membered ring (Figure 1.7).

1.5.5 Carbapenems

This class of bicyclic β-lactams is characterised by an extremely broad-spectrum of activity against both aerobic and anaerobic bacteria. The first of these compounds, thienamycin, was discovered in 1978 as a natural product of *Streptomyces cattleya* (Kahan *et al.*, 1979). Unfortunately, it was also found to be extremely unstable and therefore was not clinically developed. However, subsequent work resulted in the N-formimidoyl derivative of thienamycin, imipenem, which proved to be far more stable than thienamycin but retained its broad-spectrum of activity (Birnbaum *et al.*, 1985). Unfortunately, imipenem is hydrolysed by a dehydropeptidase enzyme located in the brush border of the mammalian kidney and consequently must be administered in combination with the enzyme inhibitor cilastatin which prevents both renal metabolism and nephrotoxicity (Kahan *et* al., 1983).

1.5.5.1 Structure of the carbapenems

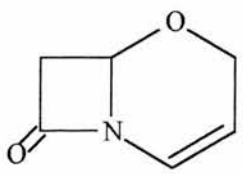
The nuclear structure of the carbapenems differs from the penam nucleus of penicillins in having a carbon atom instead of a sulphur at position 1 (Figure 1.7). They also have an unsaturated bond between C_2 and C_3 in the thiazolidine ring. An important aspect of the carbapenems is found in the stereochemistry of the side-chain linked to the β-lactam ring. In contrast to the penicillins and cephalosporins, where this group is in the cis (β) configuration, in the carbapenems, it is in the trans (α) configuration which confers striking resistance to the majority of β-lactamases.

Since the development of imipenem, subsequent research has resulted in derivatives which are more stable than their counterpart (Hikida *et al.*, 1991; Kurihara *et al.*, 1992). Meropenem is one such compound. It has the same configuration as imipenem, both have low molecular weights, are zwitterionic and penetrate bacteria readily (Trias and

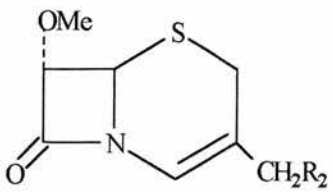
Nikaido, 1990a). However, meropenem offers several advantages in that it possesses a methyl group in position 1 which confers stability to renal dehydropeptidase I (Figure 1.7). It also has a unique side-chain in position 2, resulting in improved activity against *P. aeruginosa* and other Gram-negative bacteria (Moellering *et al.*, 1989).

Figure 1.7 Skeletons of β-lactams related to the penicillins and cephalosporins

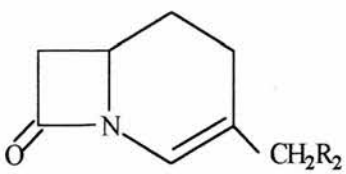
Oxacephems



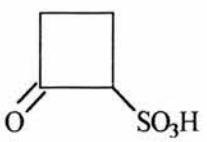
Cephamycins



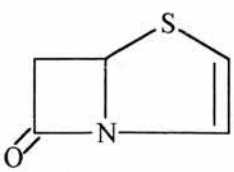
Carbacephems



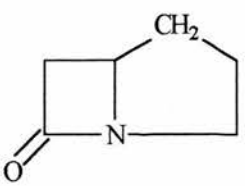
Monobactams



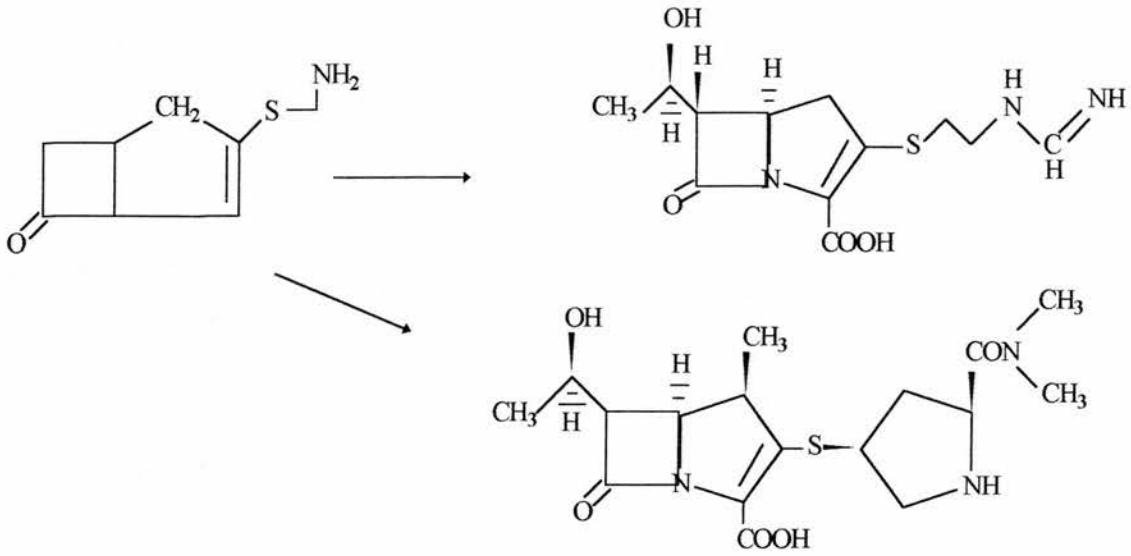
Penems



Carbapenams



Carbapenem **Imipenem**



Meropenem

1.6 Mode of action of the β-lactams

1.6.1 The bacterial cell wall - target of the β-lactams

The activity of the β-lactams demonstrates rather eloquently Paul Ehrlich's principle of 'selective toxicity' in that they act on a structurally unique feature found in both Gram-negative and Gram-positive bacteria that is absent from mammalian cells. This feature is the cell wall which provides the organism with a chemical and physical barrier against substances that may damage the cell. The major component of the cell wall is the polymer peptidoglycan which plays a vital role in providing rigidity and strength to this structure. β-lactam antibiotics act by interfering with the biosynthesis of peptidoglycan, specifically targeting the enzymes involved in the final stages of its assembly (Greenwood, 1995).

1.6.2 Structure of the cell wall

The cell wall of Gram-negative bacteria differs in structure from that of Gram-positive bacteria. Essentially, they each possess peptidoglycan, but the wall of Gram-positive organisms is a thicker, less complex structure consisting of a network of cross-linked peptidoglycan interspersed with teichoic acid and teichuronic acid polymers, the amounts of which vary between species (Neidhardt *et al.*, 1990a).

Gram-negative bacteria have a much thinner peptidoglycan layer, which is surrounded by a lipid bilayer outer membrane. This extremely efficient barrier consists of lipopolysaccharide, protein and phospholipid, all of which prevents many hydrophilic and some hydrophobic molecules from penetrating the cell. Nutrients required by the cell are able to gain entry through this barrier via protein molecules called porins that are located within the outer membrane (Neidhardt *et al.*, 1990a). These channels are also utilised by the hydrophilic β-lactam antibiotics to gain access to their target enzymes located within the periplasmic space (Nikaido, 1993). Differential activity of the β-lactams is dependant on their ability to move through the porin channels, which in turn is determined by their size and physicochemical structure (Livermore, 1987a).

Peptidoglycan is composed of linear chains of alternating β 1-4 linked N-acetyl glucosamine and N-acetylmuramic acid residues that are crossed-linked via oligopeptide bridges attached to the muramic acid residues (Neidhardt *et al.*, 1990b). Assembly consists of the synthesis of pentapeptide precursors in the cytoplasm that are subsequently transported across the cell membrane where they are added to the existing peptidoglycan chain. There are a number of enzymes involved in the final cross-linking process (Barnikel *et al.*, 1983). Transpeptidases catalyse the formation of a peptide bond between the carboxyl of the penultimate D-alanine of one pentapeptide, and the amino group of the middle amino acid of another pentapeptide. The energy for this reaction is

provided by the loss of the second pentapeptides's terminal D-alanine which is catalysed by carboxypeptidases (Barnikel *et al.*, 1983).

1.6.3 Penicillin-binding proteins

Both transpeptidases and carboxypeptidases, along with endopeptidases (which cleave peptide bonds formed between pentapeptides), are the targets of β-lactam antibiotics (Waxman and Strominger, 1983). They are also collectively known as penicillin-binding proteins (PBPs) since they are able to efficiently bind penicillin (Georgopapadakou and Liu, 1980). PBPs 1a, 1b, 2 and 3 of Gram-negatives have essential functions in peptidoglycan synthesis (Zimmerman, 1980).

Numbered in descending order according to their molecular weight, their numbers vary between species (Georgopapadakou and Liu, 1980). β-lactams exert their effect by acting as substrate analogues for the PBPs. The amide group (O=C-N) of the β-lactam ring is similar in conformation to the carboxy terminal of the D-alanyl-D-alanine. The PBPs thus effectively recognise the antibiotic as they would their normal substrate, allowing it to interfere with cell wall formation by commandeering the active-site cavity (Tipper and Strominger, 1965).

A common feature shared among the PBPs is a C-terminal transpeptidase domain with a conserved active-site serine residue that is acylated by the β-lactams resulting in the formation of a stable covalent ester, and inactivation of the enzyme (Ghuysen, 1988). The effect of this interaction on the cell depends on the enzyme involved. Penicillin binding proteins 1, 2 and 3 are transpeptidases to which most β-lactams bind (Spratt, 1977).

1.7 Bacterial resistance to antibiotics

1.7.1 The origins of resistance

Antibiotic resistance has evolved in parallel with the advent of antimicrobial chemotherapy, the first β-lactamase was identified before the widespread use of penicillin (Abraham and Chain, 1940). Given the fact that many antibiotics in use today were discovered in soil micro-organisms, it is hardly surprising that bacteria have developed a remarkable ability of acquiring resistance to them.

There is much evidence to suggest that the most likely sources of resistance genes found in clinical isolates are from the soil micro-organisms which produce many of the antibiotics in use today (Benveniste and Davis, 1973; Thompson and Gray, 1983). However, there are other potential sources, for example, modification of genes encoding essential metabolic enzymes, also known as 'housekeeping' genes (Shaw *et al.*, 1992; Rather *et al.*, 1993).

1.7.2 The role of antibiotics in producer organisms

A number of theories exist which attempt to explain the function of antibiotics in producer micro-organisms. One such theory is that they have a regulatory role during the transition from vegetative cell to spore in sporulating organisms (reviewed by Demain, 1974). A competition hypothesis also exists, which describes a functional role in the survival of producing organisms, thus offering a competitive advantage to the cell (Brian, 1957). It has even been suggested that antibiotics act as bacterial pheromones in promoting the transfer of plasmids between different bacteria (Mazodier and Davies, 1991). What *is* known is that antibiotics are secondary metabolites that are produced after the logarithmic growth phase of the organism, and that the enzymes involved in antibiotic biosynthesis are repressed during this growth period (Demain, 1974).

1.7.3 Self-defence mechanisms in antibiotic-producing micro-organisms - the origins of bacterial resistance?

Bacteria that produce potentially toxic antibiotics utilise a number of survival strategies to avoid self intoxication. A number of organisms modify the intracellular target to which the antibiotic normally binds; for example, *Streptomyces erythraeus* produces the macrolide erythromycin, which inhibits bacterial protein synthesis by binding to 50S ribosomal subunits. By methylation of its own 23S rRNA, *S. erythraeus* avoids the effects of the antibiotic (Teraoka and Tanaka, 1974). A similar methylase has been identified as the cause of resistance to erythromycin and other macrolides in *Staphylococcus aureus* and certain streptococci (Weisblum *et al.*, 1979), suggesting that a common evolutionary origin exists for the genes encoding these enzymes.

There are a number of producer organisms that possess the ability to inactivate their antibiotic products within the cell; for example, the streptomycin-producing strains of *Streptomyces griseus* and *S. bikiniensis* (Miller and Walker, 1969). During their exponential growth phase, they are normally sensitive to the drug, but once in the stationary phase, when streptomycin is actively being produced, they develop a high level of resistance. This is achieved by phosphorylation of the antibiotic to an inactive 6-phosphoryl derivative, by an enzyme, aminoglycoside 6-phosphotransferase (APH (6)), which is present in the cell at high levels during antibiotic production. Once the antibiotic reaches the cell membrane, a process of dephosphorylation occurs thereby ensuring that the drug is excreted in its active form (Nomi *et al.*, 1967).

Evidence now exists to support the hypothesis that the genes encoding phosphorylating enzymes present in clinical strains of aminoglycoside-resistant bacteria, have significant sequence homology with those present in producer bacteria (Thompson and Gray, 1983).

1.7.4 Selective pressure and antibiotic resistance

It is universally accepted that the use of antimicrobial agents, both in clinical medicine and as food supplements in animal populations, exerts a strong selective force for the emergence of bacterial resistance (Tenover and McGowan, 1996; Spika *et al.*, 1987). Particular areas within the hospital setting, for example, intensive therapy units, where bacteria are constantly being exposed to a fluctuating antibiotic environment, allow for the selection of resistant variants that are maintained and transferred within these areas (Saunders, 1981).

Intensively farmed animals, for example, poultry and pigs, act as a reservoir of antibiotic-resistant bacteria (Linton, 1986). If the foodstuff is contaminated during slaughter, these resistant bacteria pose a potential threat to human health via a number of routes (Piddock, 1996). There is evidence to suggest that an increased use of antibiotics as growth promotors and as treatment for infections in these animals, correlates with a similar rise in the number of isolated antibiotic-resistant strains (Levy *et al.*, 1987).

In addition to these factors, there are other selective pressures at play, both intrinsic to the bacteria and to the patient, which contribute to the complexity of the resistance problem (Toltzis, 1995).

1.8 Acquisition of resistance genes - the genetic vehicles of bacterial resistance

Bacteria can be intrinsically resistant to antibiotics, or they can acquire resistance by a number of mechanisms.

1.8.1 Intrinsic resistance

Intrinsic or 'natural' resistance as it is often referred to, is found to some extent in most bacterial species (Costerton & Cheng, 1975; Li *et al.*, 1994a; Li *et al.*, 1994b). It is a term usually used to describe the inherent features of a species or genus that are responsible for preventing access of the antibiotic to the interior targets of the cell (Nikaido & Nakae, 1979). It can pose a therapeutic problem for infections caused by certain bacteria such as *Acinetobacter* species (Fass & Barnishan, 1980) and *P. aeruginosa* (Yoshimura & Nikaido, 1982), both of which are multi-resistant pathogens that are frequently isolated from ITUs.

More recently, multidrug efflux pumps that are capable of pumping a wide range of compounds including antibiotics, out of the cell, have been highlighted as playing an increasingly important role in the intrinsic mechanisms of antibiotic resistance in many bacteria (Nikaido, 1996).

1.8.2 Acquired resistance

In contrast to intrinsic resistance where all the cells within a given population express the resistance phenotype, acquired resistance occurs when resistant strains emerge from previously sensitive bacterial populations, usually after exposure to antibiotics (Tenover and McGowan, 1996). It can occur as a result of mutations to the existing genome (Heisig *et al.*, 1993), or when genetic information is transferred between bacterial cells by the mechanisms of transduction, transformation and conjugation (Mazodier and Davies, 1991).

Transduction involves the transfer of DNA between bacterial cells via a bacteriophage vector, and may be restricted to related species due to the specificity involved in cell invasion by the phage (Mazodier and Davies, 1991). Transformation is an important

mechanism of DNA transfer in a few bacteria and involves the incorporation of short DNA fragments by homologous recombination in the recipient cell (Spratt *et al.*, 1992). Conjugation requires physical contact between two cells and is the mechanism of self-transfer utilised by plasmids and transposons; two genetic elements that play an important role in the transfer of resistance genes between bacteria (Watanabe, 1963; Courvalin and Carlier, 1986).

1.8.3 The vectors of acquired resistance

1.8.3.1 Plasmids

Plasmids are circular, extrachromosomal genetic elements that are able to replicate independantly of the chromosome and encode a number of functions (Lederberg, 1952). They began to attract a great deal of interest following the discovery of plasmid-mediated antibiotic resistance in Japan in the late 1950s, and the demonstration of transferable multiple drug resistance (Watanabe, 1963). At around the same time, it was discovered that penicillinase synthesis by *S. aureus* was a plasmid-mediated characteristic (Novick, 1963). Indeed, a large number of β-lactamases are now known to be plasmid-encoded, and this is undoubtedly the main reason why this mechanism of β-lactam resistance is a major contributor to the antibiotic resistance problem (Bush *et al.*, 1995).

Plasmids owe much of their success as vehicles of antibiotic resistance, to a number of factors:

1. They allow the genes that they carry to evolve independantly of the chromosome, and are therefore free of the genetic constraints that this entails.
2. They offer a high degree of mobility so that genes can be passed vertically from mother cells to progeny but can also be acquired horizontally from other bacteria.
3. They are also frequently vectors of other genetic determinants (transposable elements) involved in antibiotic resistance.

1.8.3.2 Transposable elements

The discovery of insertion sequences, transposons and more recently, integrons, has gone a long way to help explain many of the mechanisms by which bacteria acquire (and lose) resistance genes. Bacteria of the pre-antibiotic era contained plasmids very similar to those which now carry resistance genes (Hughes and Datta, 1983). Indeed, it is now known that transposable elements have been instrumental in the evolution of antibiotic resistance in bringing about important rearrangements in bacterial DNA (Saunders, 1984). A common feature of transposable elements is their ability to insert (transpose) into different sites on the same, or on different DNA molecules without the need for homology between the element and the sites of insertion. Thus, the recipient replicon can be in another microorganism (Bennett and Howe, 1990).

1.8.3.2.1 Insertion sequences and transposons

Insertion sequences (ISs) were first identified in mutants of *E. coli*, in which the mutation was due to the insertion of small pieces of DNA, rather than a classical deletion or point mutation (Jordon *et al.*, 1968). Similar elements were subsequently discovered (Fiant *et al.*, 1972), and are now known to be normal components of most bacterial chromosomes and plasmids (Galas and Chandler, 1989). ISs have been implicated in antibiotic resistance by creating novel promoters for antimicrobial resistance genes (Podglajen *et al.*, 1994).

There are several classes of transposons grouped according to their method of transposition and their gene composition (Berg, 1989; Sherratt, 1989; Phillips and Novick, 1979; Clewell and Gawron-Burke, 1986). Many carry antibiotic resistance genes in addition to those required for transposition (Saunders, 1984). Unlike plasmids, transposons are unable to replicate independantly and so must be maintained within a functional replicon that subsequently acts as a vector in transferring the transposon into a

new host. If the vector is unable to replicate within the host, the transposon can still exist by transposing itself to the host chromosome or resident plasmid (Saunders, 1981).

1.8.3.2.2 Integrons

It is now known that a considerable number of antibiotic resistance genes found in Gram-negative bacteria are part of small, mobile elements called gene cassettes (Recchia and Hall, 1995), which are normally integrated by site-specific recombination in another element commonly associated with them, called an integron (Stokes and Hall, 1989). Together, these elements form an extremely efficient natural cloning and expression system that is utilised by bacteria to disseminate resistance genes.

To date, there are over 40 cassettes that are known to contain antibiotic resistance genes, including those which confer resistance to the β-lactams (Recchia and Hall, 1995). An important feature of this recombination system is that integration of the gene cassette within the integron is reversible, and therefore captured genes can be released and subsequently recaptured by another integron (Collis and Hall, 1992). This can result in the formation of a large number of different integrons each containing a set of 1 or more cassettes.

There are 3 classes of integrons known to date (Recchia and Hall, 1995; Tietze *et al*, 1987; Young *et al.*, 1994; Arakawa *et al.*, 1995). The most prevalent integrons found in Gram-negative clinical isolates belong to class 1 which have contributed greatly to the emergence of multi-resistant Gram-negative bacteria (Martinez-Freijo *et al.*, 1998).

1.9 Mechanisms of resistance to the β-lactams

There are essentially 4 main mechanisms that bacteria utilise to evade the action of β-lactam antibiotics:

1. Changes in outer membrane permeability
2. Efflux pumps
3. Modification of PBPs
4. Production of β-lactamases

1.9.1 Outer membrane permeability

The presence of porin channels in the outer membrane of Gram-negative bacteria facilitates the transmembrane diffusion of hydrophilic molecules, including the β-lactams. (Yoshimura and Nikaido, 1985). However, mutations resulting in the loss of specific porins can result in an increase in resistance to the β-lactams. This is evident with *Pseudomonas aeruginosa*, a pathogen renowned for its intrinsic resistance to a wide range of antibiotics (Yoshimura and Nikaido, 1982). Indeed, the overall outer membrane permeability of this organism is between 12 to 100-fold lower than that of *E. coli* (Nikaido and Hancock, 1986).

Increased resistance to the β-lactams has also been attributed to a decrease in the number of porins produced by some bacteria (Yamazaki *et al.*, 1989), and the production of porins with small channels (Sato and Nakae, 1991). *Acinetobacter* spp. have improved on this by producing a decreased number of small-sized porins (Sato and Nakae, 1991). Loss of porin expression in clinical isolates of *Klebsiella pneumoniae* from patients undergoing antibiotic therapy has been attributed to the insertion of ISs (Hernández *et al.*, 1999).

Reduced permeability alone is usually insufficient to prevent antibiotic influx and subsequently, it generally occurs in combination with a second mechanism to produce this effect (Nikaido, 1981). In the case of β-lactams, this additional mechanism is usually the production of a β-lactamase (Hancock and Bell, 1988), although there is now evidence that active efflux systems also play an important role.

1.9.2 Efflux pumps

Studies suggest that the outer membrane and specific multidrug efflux pumps of Gram-negative bacteria act together to lower both the cytoplasmic and periplasmic concentrations of β-lactams, including the carbapenems (Li *et al.*, 1994b; Masuda *et al.*, 1995; Köhler *et al.*, 1999). Many of these systems are multidrug efflux pumps because they have extremely broad specificities and are capable of pumping out a wide range of compounds (Nikaido, 1994).

1.9.3 Alterations in PBPs

This mechanism of resistance is more common in Gram-positive bacteria (Brown and Reynolds, 1980), and in certain fastidious Gram-negatives such as *Haemophilus influenzae* (Serfass *et al.*, 1986). It is generally not regarded as a primary resistance mechanism in other Gram-negative bacteria that have more efficient mechanisms at their disposal. It is likely that in bacteria in which this resistance mechanism occurs, the development of β-lactam resistance is due to multiple amino acid substitutions, each resulting in a relatively small decrease in affinity of the PBP for the β-lactam antibiotic(s), and as this may potentially alter the stability of the enzyme, 'restabilizing' amino acid substitutions may also occur (Hedge and Spratt, 1985). Reduced affinity of specific PBPs has been documented for *Pseudomonas aeruginosa* (Gotoh *et al.*, 1990) and for *Acinetobacter baumannii* (Gehrlein *et al.*, 1991). β-lactam resistance can also occur as a

result of the production of additional PBPs, as seen with PBP2' (Cookson and Phillips, 1990) and PBP2B (Komatsuzawa *et al.*, 1999) of methicillin resistant *S. aureus*, or due to overproduction of a PBP possessing slow enzyme-β-lactam kinetics (Fontana *et al.*, 1983).

1.9.4 Production of β-lactamases

By far the most successful mechanism of antibiotic resistance at the disposal of bacteria is the production of β-lactamases, enzymes that can cleave the amide bond of the β-lactam ring thus rendering inactive a vast number of these antibiotics (Medeiros, 1984). β-lactamases are produced both by Gram-negative and Gram-positive bacteria, and their impact on antimicrobial efficacy has been immense. Although the advent of β-lactamase inhibitors and extended-spectrum β-lactams have appeared to restrain the effects of many of these enzymes, the bacterial response has been to modify existing β-lactamases or to produce novel, more efficient ones. This unrelenting challenge on antimicrobial therapy appears to be far from exhausted.

1.10 The evolution of β-lactamases

The hypothesis that β-lactamases have evolved from PBPs is now generally accepted as a result of sequence-based analyses, and structural and enzymatic information that has provided an understanding of this evolutionary process. We now know that β-lactamases and PBPs share several highly conserved amino acid sequences (Massova and Mobashery, 1998). As a primary defensive response to antibiotics produced by other soil bacteria, it is thought that non-producing bacteria excreted their PBPs that were bound to the antibiotic, acting in effect as a detoxification mechanism. Over time, they developed into enzymes that were capable of hydrolysing the β-lactam, thus providing these bacteria with a distinct survival advantage (Kelly *et al.*, 1986). This evolutionary process

continues today, the only difference being that the selective pressure is now the heavy use of antibiotics in the hospital setting.

1.10.1 Structure and mechanism of action

β-lactamases can be divided into 2 broad categories based on the structure of their active-site. The first category comprises those with a serine amino acid at this site, and can be further subdivided into molecular classes A, C and D on the basis of sequence similarity (Bush *et al.*, 1995). The second category comprises the small but rapidly growing group of class B β-lactamases, or metallo-enzymes, so called because they utilise 1 or 2 zinc ions to disrupt the β-lactam ring rather than the serine ester mechanism of serine-based β-lactamases (Valladares *et al.*, 1997; Carfi *et al.*, 1998).

1.10.2 Structure of the serine β-lactamases

X-ray studies have revealed structural analogies between the DD-peptidase PBP of *Streptomyces* R61 (S. R61) and several class A and C β-lactamases (Kelly *et al.*, 1986; Lobkovsky *et al.*, 1993). Essentially, these enzymes consist of 2 structural domains (the α and α/β domains), with the active-site located in a groove between the two. Class C β-lactamases and the S. R61 PBP have additional loops and secondary structures away from the active site on the surface of the α domain (Matagne *et al.*, 1998). The active-site is located at the N-terminus of the first hydrophobic helix (the α-2 helix) of this domain.

There are a number of conserved sequences at the active-sites of serine β-lactamases that play a role in substrate recognition and catalysis. The Ser-Xaa-Xaa-Lys (Xaa is any amino acid) motif contains the active-site serine residue. The side chain of the lysine residue points onto the active-site, and the amino group of this side chain is thought to be involved in the catalytic process (Knox and Moews, 1991).

The second element is referred to as the SDN loop in class A β-lactamases and almost always consists of Ser-Xaa-Asn (serine-any amino acid-asparagine) in these enzymes and most PBPs (Bush, 1997). In class C and D β-lactamases, and a few PBPs, the serine residue is replaced by Tyr (tyrosine). This motif is found in the α domain and forms one side of the active-site cavity (Bush, 1997).

The third element is called the KTG triad (Joris *et al.*, 1988), is highly conserved and forms the opposite wall of the cavity. In the majority of enzymes, it consists of the sequence Lys-Thr-Gly (lysine-threonine-glycine), but Lys may be replaced by His (histidine) or Arg (arginine) in a few rare cases, and Thr by Ser in some class A β-lactamases (Matagne *et al.*, 1998).

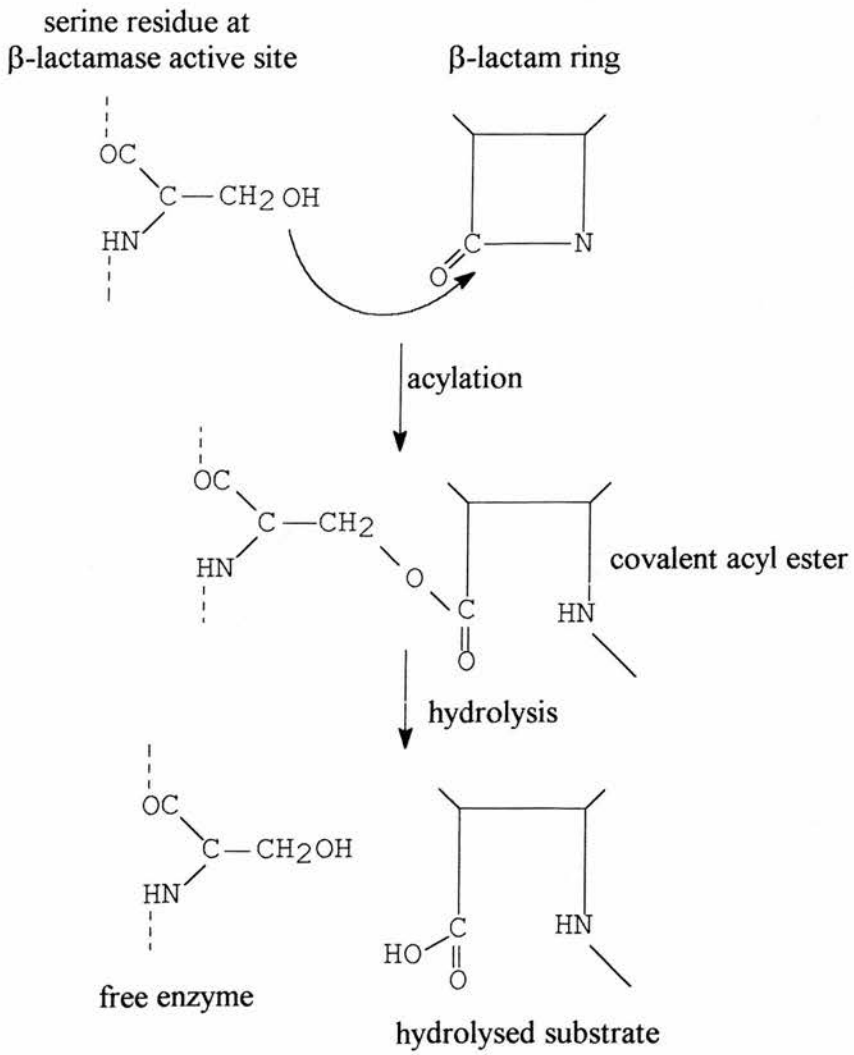
There is a fourth structure which is highly conserved in all class A β-lactamases but is altered in other active-site enzymes (Bulychev *et al.*, 1997). In class A enzymes, it is found on a 16-19 residues loop known as the Ω-loop. In most cases, this contains the sequence Glu ^{166}-Xaa-Glu-Leu-Asn ^{170} where Glutamic acid at position 166 and Asparagine at position 170 are essential in the positioning of one of the conserved water molecules which are involved in the deacylation step of the enzyme-β-lactam reaction, close to the active site (Herzberg and Moult, 1987).

1.10.3 The serine ester mechanism of class A, C and D β-lactamases

β-lactam hydrolysis begins with the non-covalent association of the β-lactamase and the β-lactam (Figure 1.8). The carbonyl group of the β-lactam ring is attacked by a free hydroxyl on the side chain of the serine residue (Ser-70 in class A and D, and Ser-64 in class C) at the active-site of the enzyme to yield a covalent acyl ester. The ester is hydrolysed, freeing the enzyme from the hydrolysed substrate. Additional residues are

known to play an important role in the formation of the acyl-enzyme intermediate, and in the regeneration of the free enzyme (Massova and Mobashery, 1998).

Figure 1.8 Mechanism of β-lactam hydrolysis utilised by serine active-site β-lactamases



1.10.4 Classification of β-lactamases

The classification of Sawai in 1968 discriminated penicillinases from cephalosporinases based on their response to antisera (Sawai *et al.*, 1968). As more information on enzyme function became available and with the discovery of new β-lactamases, other classification schemes evolved (Richmond and Sykes, 1973; Sykes and Matthew, 1976; Mitsuhashi and Inoue, 1981; Bush, 1989a; Bush, 1989b; Bush, 1989c). Amber was the first to propose a molecular classification scheme (Ambler, 1980) which is still the most straightforward.

1.10.4 1 The Ambler classification scheme

When Ambler first proposed a molecular structure classification, only four β-lactamase amino acid sequences were known (Ambler, 1980). On that basis, there are now four molecular classes to which β-lactamases are assigned based on the similarity of their amino acid sequences (Jaurin and Grundström, 1981; Huovinen and Jacoby, 1991; Ouellete *et al.*, 1987). Classes A, C, and D contain the serine active-site β-lactamases and represent the majority of known enzymes. Class A enzymes share considerable homology and include TEM-1 and SHV-1, which are two of the most prevalent β-lactamases found in Enterobacteriaceae (Wiedemann *et al.*, 1989). Many class A β-lactamases are encoded by genes on plasmids or transposons, and are produced by both Gram-positive and Gram-negative bacteria (Bush *et al.*, 1995). Fewer class C β-lactamases have been found associated with transmissible elements, and they are only produced by Gram-negative bacteria (Bush *et al.*, 1995). Class B comprises the metallo-β-lactamases which require a bivalent metal ion (usually zinc) for their activity, and class D are a group of enzymes which are capable of hydrolysing oxacillin (Dale *et al.*, 1985) that appear to have evolutionary links with high molecular-weight class C PBPs (Massova and Mobashery, 1998).

1.10.4.2 The Bush classification scheme

The most recent scheme of Bush attempts to correlate both the phenotypic and the molecular properties of known β-lactamases (Bush *et al.*, 1995). It refrains from using the location of the gene encoding the β-lactamase as a primary classification factor, reflecting the ability of a large number of these genes to move between chromosome and plasmid. There are four main functional groups within this classification scheme.

Group 1

Chromosomal cephalosporinases of this group are often referred to as AmpC-type β-lactamases that are intrinsically resistant to β-lactamase inhibitors and are ubiquitous in most enterobacteria. The amount and mode of expression of these enzymes vary between genera (Sanders and Sanders, 1988). Some of the genes encoding these enzymes have migrated onto plasmids in *Klebsiella pneumoniae* and *Escherichia coli* clinical isolates (Sanders and Sanders, 1992). Of concern is the recent report of a plasmid-mediated AmpC β-lactamase (ACT-1) in *K. pneumoniae* which, in combination with loss of an outer membrane protein resulted in resistance to imipenem (Bradford *et al.*, 1997).

Group 2

The β-lactamases of this group belong to molecular classes A or D and are generally inhibited by active-site-directed inhibitors. They hydrolyse a diverse range of substrates which is acknowledged by the definition of subgroups for these enzymes. Extended-spectrum β-lactamases that are derived from the TEM and SHV enzymes (group 2b) constitute group 2be, and those that have a reduced affinity for β-lactamase inhibitors comprise the 2br group. The main criterion for group 2d is the preferential hydrolysis of cloxacillin or oxacillin (hydrolysis rate of greater than 50% than that for

benzylpenicillin) (Bush *et al.*, 1995). Likewise, β-lactamases that hydrolyse carbenicillin at a rate of greater than 60% compared with that for benzylpenicillin have been allocated to group 2c. Group 2f comprises the active-site serine carbapenemases. Many of the genes that encode these β-lactamases are found on plasmids.

Group 3

The metallo-β-lactamases of this group are not inhibited by classical β-lactamase inhibitors with the exception of EDTA, a metal chelator and p-chloromercuribenzoate (p-CMB), an amino-acid modifier.

Group 4

Penicillinases that are not inhibited by the suicide inactivator clavulanic acid, have been placed in this group. The LCR-1 β-lactamase from *P. aeruginosa* was originally a member of group 4 (Bush, 1989c) however, sequence data has subsequently revealed its homology with class D OXA enzymes (Couture *et al.*, 1992) with the result that it has been relocated to group 2d (Bush *et al.*, 1995).

Bush and colleagues have endeavoured to produce a "workable, and potentially useful, compilation of β-lactamase characteristics" however, as they concede, "no functional classification will ever be completely satisfactory" (Bush *et al.*, 1995). As more sequence data is revealed about β-lactamases, and enzyme variants emerge with altered substrate and inhibitor profiles as a result of point mutations of their genes, it is likely that the composition of the classification groups described here will alter accordingly.

1.11 Mechanisms of resistance to the carbapenems

The advent of the carbapenems was embraced with a sense of great expectation in that at last, a class of β-lactams now existed that seemed capable of evading many of the resistance mechanisms which have compromised the use of other β-lactams. However, cases of resistant clinical isolates have begun to emerge and initially, resistance mechanisms other than the production of β-lactamases were considered solely responsible. Impermeability mechanisms have been shown to play a role in a number of Gram-negative bacteria (Buschner *et al.*, 1987; Pagani *et al.*,1990; Mehtar *et al.*, 1991), as has PBP modification (Neuwirth *et al.*, 1995; Gehrlein *et al.*, 1991), although the importance of the latter appears to be of little consequence in Gram-negative bacteria due to the ability of the carbapenems to target the vulnerable site of PBP2 (Hashizume *et al.*, 1984).

There are now an increasing number of reports that attribute carbapenem resistance to β-lactamase activity. In many of these cases, a change in outer membrane permeability is also required, but there is now a growing number of so called 'efficient carbapenemases' that appear to be able to cause carbapenem resistance on their own.

1.11.1 Outer membrane impermeability and Bush group 1 β-lactamase production

There is a specific porin protein, OprD, in *P. aeruginosa* that is used by the organism to bring dipeptides into the cell (Hancock and Bell, 1988). The zwitterionic carbapenems are sufficiently similar in structure to these dipeptides that they are also able to enter the cell by this route via the same binding site (Huang and Hancock, 1993; Trias and Nikaido, 1990a). However, imipenem resistance has occurred in this organism as a result of loss of this porin (Buschner *et al.*, 1987). It has subsequently been reported that this

mechanism can function only when a chromosomal β-lactamase is expressed (Livermore, 1992). Many of these particular β-lactamases have been demonstrated to hydrolyse the carbapenems albeit at a very slow rate (Livermore and Yang, 1987b). OprD-deficient clinical isolates of *P. aeruginosa* with derepressed production of their chromosomal β-lactamase have been found to be four-fold more resistant to imipenem than OprD-deficient isolates that only produced basal levels of the enzyme (Satake *et al.*, 1991).

Expression of chromosomal β-lactamase does not appear to have a role in meropenem resistance in laboratory mutants lacking OprD (Livermore, 1992). Similar findings have been demonstrated in *Enterobacter cloacae* (Cornaglia *et al.*, 1995). Interestingly, *P. aeruginosa* mutants overexpressing the MexAB-OprM efflux pump have demonstrated an increase in meropenem resistance but not in imipenem resistance (Masuda and Ohya, 1992). Studies have revealed that meropenem (but not imipenem) is a substrate of this pump in *P. aeruginosa*, and it has been suggested that differences in the physicoproperties of the two antibiotics may explain this observation (Köhler *et al.*, 1999). Alternative ports of entry have also been suggested for meropenem in *P. aeruginosa* (Perez, F. J. *et al.*, 1996).

The interplay of overexpression of group 1 β-lactamases and outer membrane impermeability appears to be the main mechanism of carbapenem resistance in *Enterobacter* spp. (Hopkins and Towner, 1990; Chow and Shlaes, 1991; Raimondi *et al.*, 1991; Lee *et al.*, 1991; De Champs *et al.*, 1993; Cornaglia *et al.*, 1995). It has also been reported in *Citrobacter freundii* (Marinardi *et al.*, 1997), in *Proteus mirabilis* (Mehtar *et al.*, 1991), and in *Providencia rettgeri* (Raimondi *et al.*, 1991). The decrease in permeability is thought to slow the rate of entry of the carbapenem sufficiently enough to permit the large amount of β-lactamase present in the periplasmic space to inactivate the drug before it reaches its target site on the cytoplasmic membrane (Raimondi *et al.*, 1991). More recently, there has been a report of carbapenem resistance in a clinical

isolate of *E. coli* associated with production of a plasmid-determined molecular class C β-lactamase in combination with loss of an outer membrane protein (Stapleton *et al.*, 1999). In addition, the production of an extended-spectrum β-lactamase SHV-2, along with the loss of an outer membrane protein, has been reported as a mechanism of imipenem resistance in a clinical isolate of *K. pneumoniae* (Mackenzie *et al.*, 1997). This observation has subsequently been described in another clinical isolate of the same species but has been attributed to an increased inoculum effect (Martínez-Martínez *et al.*, 1999).

1.11.2 Efficient carbapenemases

The molecular class B metallo-β-lactamases and some serine active-site β-lactamases are both able to hydrolyse the carbapenems, and are often referred to as carbapenemases.

1.11.2.1 The metallo-β-lactamases

The chromosomally-encoded zinc-dependant β-lactamase from *Bacillus cereus* was the first metallo-β-lactamase to be reported (Sabath and Abraham, 1966). A number of similarly encoded metallo-β-lactamases subsequently emerged in *Pseudomonas* (now *Stenotrophomonas*) *maltophilia* (Saino *et al.*, 1982), *Aeromonas hydrophila* (Shannon *et al.*, 1986), *Bacteroides fragilis* (Cuchural *et al.*, 1986), *Legionella gormanii* (Fujii *et al.*, 1986) and *Flavobacterium odoratum* (Sato *et al.*, 1985). Although these enzymes were greeted with interest, they were considered to be of little clinical significance since they were recovered from single isolates. However, there have since been numerous reports of other metallo-enzymes from these and other species (Rasmussen and Bush, 1997; Stunt *et al.*, 1998; Walsh *et al.*, 1996; Woodford *et al.*, 1998). Of greater concern, are the reports of plasmid-mediated metallo-β-lactamases. The first of these, from *P. aeruginosa*, was reported in 1991 (Watanabe *et al.*, 1991), followed closely the following year by the report of a transferable metallo-enzyme from *B. fragilis* (Bandoh *et al.*, 1992). They have subsequently been joined by a plethora of

extra-chromosomally-encoded metallo-enzymes (Cardoso *et al.*, 1999; Bellais *et al.*, 1999; Rasmussen and Bush, 1997; Lauretti *et al.*, 1999).

From a clinical viewpoint, IMP-1 is considered to be the most threatening metallo-β-lactamase, since it has succeeded in becoming established in several species of enterobacteria and in *P. aeruginosa* in Japan. It was originally isolated in Japan from a clinical strain of *Serratia marcescens*, and was found to be chromosomally-encoded, conferring resistance to imipenem and broad-spectrum β-lactams (Osano *et al.*, 1994). It shares similar kinetic properties and identical sequence with the metallo-enzyme described by Watanabe *et al* in 1991, with the result that the two are now considered to be the same (Iyobe *et al.*, 1996).

The acquisition of IMP-1 does not necessarily coincide with the emergence of high-level carbapenem resistance (Senda *et al.*, 1996). An additional factor, most likely impermeability, has been suggested for resistance to occur in some cases (Minami *et al.*, 1993).

An alarming development has been the discovery of the gene encoding IMP-1 (bla_{IMP}) on an integron-like element (Arakawa *et al.*, 1995). In addition, the integrons carrying bla_{IMP} also possess the aminoglycoside acetyltransferase gene (*aac*(6')-Ib), so that cross-resistance can also occur to amikacin, tobramycin and netilmicin (Arakawa *et al.*, 1995). Fears of this facilitating the spread of the IMP-1 gene to other bacteria, both within and outside of Japan are now being realised. It has already been found, amongst others in clinical isolates of *E. coli, K. pneumoniae, C. freundii,* and *Burkholderia cepacia* (Kurokawa *et al.*, 1999), and appears to be disseminating, with reports of IMP-1-producing clinical isolates in Singapore (Koh *et al.*, 1999). An IMP-1-like β-lactamase has recently been discovered in Italy from a clinical isolate of *Acinetobacter baumannii* (Cornaglia *et al.*, 1999).

There is no doubt that the heavy use of carbapenems and other antibiotics in Japan has exerted a strong selective pressure that has brought about the emergence of strains producing IMP-1 and other related metallo-β-lactamases (Livermore, 1997), and that a reduction in the use of these antibiotics results in a concomitant decrease in the number of strains which produce such enzymes (Senda *et al.*, 1996). However, there is still the possibility that silent genes remain in these bacteria, as observed in *B. fragilis* (Podglajen *et al.*, 1992), that can be switched on following a return of antibiotic pressure.

1.11.2.2 Serine active-site carbapenemases

Serine active-site β-lactamases that are capable of hydrolysing the carbapenems are a relatively recent addition to this type of resistance. The first, Sme-1, was isolated from two *S. marcescens* strains collected in London in 1982 (Yang *et al.*, 1990). The second, IMI-1, emerged two years later in two *E. cloacae* isolates from a hospital in southern California (Rasmussen *et al.*, 1996), and a third, NMC-A, was isolated from a single *E. cloacae* collected in Paris in 1990 (Nordmann *et al.*, 1993). Interestingly, both IMI-1 and Sme-1 were discovered before the marketing of imipenem. Although all three β-lactamases are chromosomally-encoded, they are not typical for their host species therefore, it can be assumed that they have been acquired and that the genes encoding them have become integrated into the chromosome (Yang *et al.*, 1990; Rasmussen *et al.*, 1996; Nordmann *et al.*, 1993). Consequently, the possibility exists in the future that the genes encoding these β-lactamases may once again find their way onto transposable elements if there is sufficient selection pressure.

Serine active-site carbapenemases differ from metallo-β-lactamases in a number of ways. The most obvious is in their classification. Sme-1, IMI-1 and NMC-A are all molecular class A enzymes, and belong to Bush group 2f (Bush *et al.*, 1995). They also display a greater resistance to imipenem than meropenem, unlike the metallo-β-lactamases which produce similar resistance to both. The class A carbapenemases are also resistant to

aztreonam but not the third-generation cephalosporins, and are inhibited by clavulanic acid. Metallo-β-lactamases are not active against aztreonam, nor are they inhibited by clavulanic acid (Livermore, 1997).

In contrast with the expression of metallo-β-lactamase genes which can be either inducible in response to the presence of a β-lactam, or non-inducible (Rasmussen and Bush, 1997), expression of the genes encoding all three class A carbapenem-hydrolysing β-lactamases is inducible and is under the control of LysR-like regulatory proteins, the genes of which are found immediately upstream of their respective β-lactamase genes (Naas and Nordmann, 1994; Naas *et al.*, 1995; Rasmussen *et al.*, 1996). All three β-lactamases have now been sequenced. Sme-1 is about 70% identical to IMI-1 and NMC-A, which both share 95% identity (Naas *et al.*, 1995; Naas and Nordmann, 1994; Rasmussen *et al.*, 1996). To date, Sme-1 and NMC-A have not been found outwith their producer isolates. However, more recently, Sme-type carbapenem-hydrolysing β-lactamases have been reported in the USA from geographically diverse isolates of *S. marcescens* which share similar substrate profiles with Sme-1, and which differ from the parent enzyme by a single amino-acid change (Queenan *et al.*, 1999).

The carbapenemase story has however, taken a rather diverse twist with *Acinetobacter* spp. recently, with an increase in the incidence of carbapenem-hydrolysing β-lactamases that are not zinc dependant. In particular, many of those reported are not class A enzymes but instead appear to be oxacillin-hydrolysing β-lactamases of molecular class D.

1.11.2.3 Proposed mechanism of carbapenem hydrolysis by serine active-site β-lactamases

Site-directed mutagenesis studies with Sme-1 have identified a possible role of the amino-acid serine at position 237 (Ser-237) in the hydrolysis of imipenem (Sougakoff *et al.*, 1999). This residue is located near the highly conserved KTG motif in the active-site of class A β-lactamases, which plays an important role in substrate binding. By replacing the serine with an alanine (Ser-237-Ala mutant), this particular study demonstrated a subsequent 5-fold decrease in imipenem hydrolysis. The mutant retained activity against aztreonam, and it was confirmed therefore that Ser-237 has no specific role in the hydrolysis of the monobactams or the oxyiminocephalosporins. Sougakoff and colleagues have suggested that Ser-237 allows a better positioning of imipenem or its acylated intermediate within the active-site of Sme-1. However, the mutant was found to retain a low but nonetheless significant activity against imipenem, which suggests the involvement of other residues at the active-site, that are needed for the efficient hydrolysis of imipenem by this enzyme.

Other studies have provided possible candidates for this role. The asparagine residue at position 132 (Asn-132) is displaced at the active-site of NMC-A, and it has been proposed that this subtle relocation enlarges the substrate-binding site to accommodate the C-6 side chain of carbapenems, allowing the turnover process to take place (Swarén *et al.*, 1998).

Another important difference observed between the group 2f carbapenemases and other class A β-lactamases is the replacement of arginine with alanine at position 244 (Arg-244-Ala). Arginine at this position is thought to interact with the C-3 carboxylate group of the substrate after acylation (Jelsch *et al.*, 1993). An alanine residue in its place may therefore alter this interaction and lead to a faster substrate turnover rate for the group 2f enzymes (Rasmussen and Bush, 1997). Interestingly, mutations at position 244

(among others) of TEM-1 and TEM-2 result in derivatives of these enzymes that are resistant to inhibitors such as clavulanic acid and sulbactam (Bush and Jacoby, 1997; Canica *et al.*, 1997).

A histidine to tyrosine change at position 105 (His-105-Tyr), and a tyrosine in place of a leucine at position 207 (Leu-207-Tyr) in the three group 2f β-lactamases may also contribute to a more effective hydrolysis rate of the carbapenems (Rasmussen and Bush, 1997). In addition, a glucose residue at position 104 in most other class A β-lactamases is found at position 103 in Sme-1, IMI-1 and NMC-A, which may place the catalytic water molecule in a more desirable position for carbapenem hydrolysis (Rasmussen and Bush, 1997).

1.12 The clinical consequences of carbapenem resistance

The selective pressure exerted by the use of broad-spectrum antibiotics in the hospital setting has resulted in a gradual shift in the pattern of pathogens that are now being isolated from patients. This is particularly evident in the intensive therapy units (ITUs) where the already difficult situation of treating immuno-compromised patients is further complicated by conditions specific to these units for example, invasive monitoring and the ventilation of patients.

Enterobacteriaceae remain major pathogens in ITUs in Europe (Vincent *et al.*, 1995), and the increased incidence of β-lactam resistance has provided a major obstacle for the successful treatment of infections caused by these bacteria (Nordmann, 1998). There has also been a resurgence in infections caused by Gram-positive bacteria that have developed resistance to many of the antibiotics which have been until now the mainstay of successful therapy (Cookson and Phillips, 1990; Hiramatsu *et al.*, 1997; French, 1998). Undoubtedly the most alarming trend is the emergence of previously clinically

insignificant bacteria as major causes of ITU-related infections. Many of these 'opportunistic' pathogens demonstrate multi-resistance (Simpson *et al.*, 1993; Denton and Kerr, 1998), and have subsequently added the carbapenems to their substrate range which have traditionally been the treatment of choice for these infections. *Acinetobacter* spp. are among this elite group of predominantly nonfermenting Gram-negative bacteria (Quinn, 1998), and are now considered a major pathogen in ITUs (Bergogne-Bérézin and Towner, 1996).

1.13 *Acinetobacter* - A taxonomic history

Acinetobacter spp. are Gram-negative coccobacilli that are strictly aerobic, non-motile, catalase-positive and oxidase-negative, and are distributed widely in nature. They are able to grow at a wide range of temperatures, and have a DNA G+C content of between 39 - 47%.

The on-going taxonomy of the genus *Acinetobacter* is complex and can be somewhat confusing. They were originally described by Beijerinck in 1911 under the name of *Micrococcus calco-aceticus* (Beijerinck, 1911). Since then, they have been included in a number of other genera including *Bacterium* (*anitratrum*) (Schaub *et al.*, 1948), *Herellea* (*vaginola*) and *Mima* (*polymorpha*) (Debord, 1939), *Achromobacter, Alcaligenes* and *Neisseria* (Juni, 1972). Two microbiologists working in France introduced the name of *Acinetobacter* for a heterogeneous group of non-motile, Gram-negative, oxidase-positive (*Moraxella)* and oxidase-negative organisms (Brisou and Prévôt, 1954) that were readily identifiable by their lack of pigmentation (Ingram and Shewan, 1960).

In 1971, the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria proposed that the oxidase-negative members alone should comprise the genus *Acinetobacter* (Lessel, 1971), following the studies by Baumann and colleagues which clearly

demonstrated that they differed from oxidase-positive strains (Baumann *et al.*, 1968). The revamped genus was included in the family Neisseriaceae, and consisted of one species (*A. calcoaceticus*) which comprised two subspecies, or varieties (var. *anitratus* and var. *lwoffii*) (Juni, 1984).

In 1986, Bouvet and Grimont identified 12 genomic species by DNA-DNA hybridization (Bouvet and Grimont, 1986). The genus is now in the new family Moraxellaceae, which also comprises the genera *Moraxella*, *Psychrobacter* and related organisms (Rossau *et al.*, 1991), and there are subsequently over 19 genomic species, of which 7 have been allocated species names (*calcoaceticus*, *baumannii*, *haemolyticus*, *junii*, *johnsonii*, *lwoffii*, and *radioresistens*). However, discrepancies remain in the numbering schemes employed by individual laboratories (Bergogne-Bérézin and Towner, 1996). A phenotypic group (*A. calcoaceticus-A. baumannii*, or Acb complex) has been proposed for genomic species 1 and 2, along with the unnamed DNA groups 3 and 13 *sensu* Tjernberg and Ursing (TU), which share DNA homology values of 67-78% (Tjernberg and Ursing, 1989).

1.14 Nosocomial infections caused by *Acinetobacter* spp.

Acinetobacter spp. are opportunistic pathogens associated with septicaemia, endocarditis, meningitis, wound and urinary tract infections and, in particular, are responsible for outbreaks of nosocomial pneumonia in ventilated ITU patients (Bowton, 1999). The most commonly isolated species from these infections is *A. baumannii* (Bergogne-Bérézin and Joly-Guillou, 1991), although other species have also been associated with serious infections (Meiki and Sramek, 1992; de Beaufort *et al.*, 1999).

Acinetobacter spp. are frequently isolated from infections along with other pathogens, particularly in the case of bacteraemia (Bergogne-Bérézin and Towner, 1996) or infected wounds (Amsel and Horrilleno, 1985), and as a result, it is often difficult to distinguish

between true infection and colonisation (Struelens *et al.*, 1993). However, ITU patients presenting with an *Acinetobacter* infection commonly incur complications that may be potentially life-threatening (Rodríguez-Baño, 1999). Acquisition of *A. baumannii* is often associated with an increased length of hospital stay and an increased mortality rate in ITU patients with proven infection (Garcia-Garmendia *et al.*, 1999).

A survey of Gram-negative aerobes isolated in ITUs in the USA between 1990 and 1993 reported that *Acinetobacter* spp. accounted for 5.3% of these organisms (Itokazu *et al.*, 1996). More recently, a similar study of ITUs in 5 European countries between 1994 and 1995 reported that *Acinetobacter* spp. were among the most frequently isolated aerobic Gram-negative bacilli (2-10%) after *E. coli*, *P. aeruginosa*, *Enterobacter* spp. and *Klebsiella* spp., with the highest numbers isolated from ITUs in France. In addition, *Acinetobacter* spp. were among those organisms that demonstrated the highest incidence of resistance in all 5 countries (Hanberger *et al.*, 1999).

1.14.1 Risk factors associated with *Acinetobacter* infections

The susceptibility of the patient is a major risk factor in the acquisition of *Acinetobacter*-related infections. ITU patients are at most risk since they are frequently ventilated, have been exposed to broad-spectrum antibiotic therapy, or have undergone invasive procedures (Gomez *et al.*, 1999). The elderly and neonates are also high risk groups (Bergogne-Bérézin and Towner, 1996; de Beaufort *et al.*, 1999). The severity of an underlying disease has also been identified as an important factor in the acquisition of *A. baumannii* infections (Bergogne-Bérézin and Towner, 1996), and an increase in mortality rate is often associated with infections caused by multi-resistant *A. baumannii* (Gomez *et al.*, 1999).

Acinetobacter spp. can be found as part of the normal flora of the human skin, especially the axillae, groin and toe webs (Somerville and Noble, 1970). However, they are also

found to a lesser degree in the respiratory tract of healthy adults (Rosenthal and Tager, 1975). Carriage is higher in hospitalised patients than in healthy, non-hospitalised individuals (Joly-Guillou and Brun-Buison, 1996), and subsequently, a high colonisation rate of the respiratory tract is thought to be associated with outbreaks involving mechanically-ventilated ITU patients (Buxton *et al.*, 1978; Koljalc *et al.*, 1999). Contact between hospital staff and patients colonised with *A. baumannii* is an important factor in the spread and maintenance of infection outbreaks (Getschell-White *et al.*, 1989).

Acinetobacter spp. are not considered to be highly virulent organisms, although they do possess characteristics common to other Gram-negative bacteria that no doubt enhance their virulence (Joly-Guillou and Brun-Buisson, 1996). They are however renowned for their ability to survive in the environment in dry conditions for prolonged periods. Environmental contamination during ITU outbreaks can be extensive and is usually in the vicinity of infected or colonised patients (Allen and Green, 1987). Contaminated bedding material and various equipment act as important reservoirs for the dissemination of *Acinetobacter* spp. (Joly-Guillou and Brun-Buisson, 1996). More recently, it has been suggested that vegetables may be a natural habitat of *A. baumannii* and may therefore provide a route by which this species is introduced into the hospital setting (Berlau *et al.*, 1999). *Acinetobacter* spp. are also able to survive exposure to certain disinfectants that are used routinely in hospitals, especially if the recommended concentration is not used (Berry *et al.*, 1990).

1.15 The resistance profile of *Acinetobacter* spp.

The ability of *Acinetobacter* spp. to develop antibiotic resistance extremely rapidly may have originated from their exposure to antibiotics in nature from soil antibiotic-producing micro-organisms. In combination with the widespread use of antibiotics in the hospital setting, this has resulted in the establishment of *Acinetobacter* as an increasingly important nosocomial pathogen with a reputation for being extremely difficult to treat.

In the 1950s and 1960s before selective pressure by broad-spectrum antibiotics had become established in hospitals, *Acinetobacter* spp. were generally resistant to penicillin and chloramphenicol, susceptible to the tetracyclines, and demonstrated variable susceptibility to streptomycin, erythromycin and sulphonamides, depending on the species (Bergogne-Bérézin, 1996). However, by the 1970s, increasing rates of resistance were observed, a trend that has continued ever since. Many acinetobacters are now resistant to the majority of the most commonly used antibiotics including the aminopenicillins, ureidopenicillins, narrow- and expanded-spectrum cephalosporins, and tetracyclines (Bergogne-Bérézin and Towner, 1996). They have also added many of the newer antibiotics to this substrate range, with resistance rates to the fluoroquinolones, broad-spectrum cephalosporins, tobramycin and amikacin on the increase (Seifert *et al.*, 1993; Vila *et al.*, 1993; Acar and Goldstein, 1997). Generally, species other than *A. baumannii* are more susceptible to antibiotics, and are less associated with nosocomial infections (Traub and Spohr, 1989; Bergogne-Bérézin and Joly-Guillou, 1985).

Until recently, the carbapenems have been effective antibiotics against multi-resistant *Acinetobacter* spp. (Seifert *et al.*, 1993; Vila *et al.*, 1993). However, imipenem resistance is now being reported with increasing frequency, particularly in *A. baumannii* strains (Go *et al.*, 1994; Tankovic *et al.*, 1994; Itokazu *et al.*, 1996; Hanberger *et al.*, 1999).

1.15.1 Antibiotic resistance mechanisms of *Acinetobacter baumannii*

Multiple antibiotic resistance in *A. baumannii* clinical isolates is due to a number of mechanisms at the disposal of this species (Table 1.1). Resistance to the carbapenems however, has generated considerable interest and concern recently, primarily due to an increasing number of reports describing β-lactamase-mediated resistance to this antibiotic class.

Table 1.1 Resistance mechanisms reported in *Acinetobacter* spp.

Antibiotic	Mechanism(s)	References
β-lactams	**penicillins** -class A β-lactamases e.g. TEM-1, TEM-2, CARB-5, SHV-derived, class D - OXA-21	Joly-Guillou *et al.*, 1998, Vila *et al.*, 1997a
	cephalosporins - class C β-lactamases e.g. ACE 1-4, PER-1	Hood and Amyes., 1989, Vahaboglu *et al.*, 1997, Poirel *et al.*, 1999
	Both - decreased outer membrane permeability	Sato and Nakae, 1991
	altered penicillin-binding proteins	Obara and Nakae, 1991
	? active efflux	Vila, 1998
	carbapenems - Altered penicillin-binding proteins, loss of OMP	Gehrlein *et al.*, 1991, Clark, 1996
	Serine β-lactamases - e.g. ARI-1, OXA-type	Paton *et al.*, 1993, Hornstein *et al.*, 1997, Afzal-Shah *et al.*, 1999b, Bou and Martínez-Beltrán, 1999
	Metallo-β-lactamases - e.g. IMP-1-type	Cornaglia *et al.*, 1999, Pérez A. N *et al.*, 1996
Aminoglycosides	aminoglycoside-modifying enzymes - Acetyltransferases (AAC) Adenyltransferases (AAD or ANT) Phosphotransferases (APH)	Lambert *et al.*, 1997
Fluoroquinolones	Mutations in *gyrA* of DNA gyrase	Vila *et al.*, 1995, Moreau *et al.*, 1996
	Mutations in *parC* of topoisomerase IV	Vila *et al.*, 1997b, Seward and Towner, 1998
	Decreased outer membrane permeability	Vila., 1998
Others -		
Chloramphenicol	Acetyltransferase I (CAT1)	Devaud *et al.*, 1982
Trimethoprim	Dihydrofolate reductase (*dhfrIa* gene)	Amyes and Young., 1996
Sulphonamides	Dihydropteroate synthese (*sul* I gene)	Vila, 1998
Tetracycline	Active efflux, ribosomal protection by soluble protein	Vila, 1998

1.15.2 Mechanisms of carbapenem resistance

A decrease in outer membrane permeability remains the main mechanism of resistance to this class in the majority of clinical strains. The outer membrane of *Acinetobacter* is less permeable than that of *P. aeruginosa*, an organism renowned for its ability to confer resistance by restricting the passage of antibiotics through its outer membrane porins (Yoshimura and Nikaido, 1982). Sato and Nakae demonstrated that the amount of porins present in an *A. calcoaceticus* strain was less than 5% of the total outer membrane protein compared with 60% in *E. coli*, and that diffusion rates of the carbapenems across the outer membrane were less than 2% of *E. coli*, suggesting that the inefficient permeability of the *A. calcoaceticus* outer membrane is due to the presence of a small number of small-sized porins (Sato and Nakae, 1991). These findings are in agreement with more recent studies involving imipenem-resistant *A. baumannii* isolates (Clark, 1996).

The PBPs of *Acinetobacter* spp. are unlike those of *E. coli* or *P. aeruginosa* and have instead a higher degree of similarity with those of the genus *Bacteroides* (Piddock and Wise, 1986). Studies investigating the roles of PBPs and carbapenem resistance in this genus are somewhat lacking however, Gehrlein and colleagues have reported an alteration of PBPs in clinical isolates of *A. baumannii* as a mechanism of imipenem resistance (Gehrlein *et al.*, 1991).

In 1985, a strain of *A. baumannii* that demonstrated resistance to imipenem was isolated from a blood culture at the Royal Infirmary of Edinburgh. When the β-lactamase content of this strain was examined, an unidentified enzyme was discovered in addition to a chromosomal cephalosporinase. Further studies revealed that this β-lactamase hydrolysed imipenem and azlocillin, which was surprising since imipenem had not been in use in the hospital at this time. The β-lactamase was subsequently named ARI-1 (***A**cinetobacter* **r**esistant to **i**mipenem) (Paton *et al.*, 1993).

Although initial conjugation studies failed to demonstrate the transfer or mobilisation of the ARI-1 gene, Scaife and colleagues later demonstrated that it was indeed plasmid-encoded by successfully transferring the gene from its original host to an *A. junii* recipient, with visualisation of the same plasmid in both donor and recipient strains (Scaife *et al.*, 1995). An unusual characteristic of ARI-1 is that it appears to be a serine active-site enzyme rather than a metallo-β-lactamase (Paton *et al.*, 1993). There have so far only been isolated reports of metallo-β-lactamases in *Acinetobacter* (Pérez, A. N. *et al.*, 1996; Cornaglia *et al.*, 1999; Da Silva and Peixe, 1999; Afzal-Shah *et al.*, 1999a).

Although the discovery of ARI-1 initially caused concern, it has not been found in any other clinical strains to date. However, a number of other β-lactamases have since been reported which have been linked to imipenem resistance (Table 1.1). The worrying trend is that the majority of these enzymes are similar to ARI-1 in that they are not metallo-β-lactamases but are instead serine active-site enzymes.

The geographical distribution of these carbapenemases is also rather worrying. *Acinetobacter* clinical isolates isolated from a burns unit in London in 1996 demonstrated reduced susceptibilities to both imipenem and meropenem, and subsequent studies revealed the presence of carbapenemase activity in these strains (Weinbren *et al.*, 1998). Subsequent reports are emerging of similar findings in isolates from Argentina, Belgium, Hong Kong, Kuwait, Singapore and Spain (Afzal-Shah *et al.*, 1998).

These findings are complicated further by the discovery of an oxacillin-hydrolysing β-lactamase from a clinical isolate of *A. baumannii* that has been linked to imipenem resistance (Hornstein *et al.*, 1997). Moreover, this does not appear to be a one-off report since similar β-lactamases have subsequently been identified in strains from Argentina (Afzal-Shah *et al.*, 1999b), and Spain (Bou and Martínez-Beltrán, 1999), and sequencing of the ARI-1 gene has revealed that this too is a molecular class D β-lactamase (Donald *et al.*, 2000). These enzymes appear to represent the next

evolutionary stage in bacterial antibiotic resistance, since this class has never before been associated with carbapenem resistance.

AIMS OF THIS THESIS

The main aims of this thesis were:

- To determine the extent of antibiotic resistance, in particular carbapenem resistance, in clinical isolates of *Acinetobacter* spp. collected world-wide.

- To identify and characterise the mechanism(s) of carbapenem resistance in resistant isolates.

- To investigate the role of fluoroquinolones in the treatment of multi-resistant *Acinetobacter* spp. and their mechanisms of resistance.

- To determine the efficacy of sulbactam as a potential therapeutic alternative against carbapenem-resistant *Acinetobacter* spp.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains

Table 2.1 contains clinical isolates of *Acinetobacter* species investigated in this study. Standard bacterial strains and standard β-lactamase-producing strains are listed in table 2.2. The API 20NE identification system (Biomérieux, France) was used for species identification.

2.2 Storage of strains

Bacterial strains were inoculated onto MacConkey agar plates and incubated at 37°C overnight. Cryovials (Alpha Laboratories, Eastleigh, Hampshire) containing 900 µl of nutrient broth (Oxoid, Basingstoke, Hampshire) were inoculated with 1 colony from the plates, the suspension was vortexed and incubated at 37°C overnight. A 100 µl volume of 50% sterile glycerol was added to each cryovial to give a final concentration of 5%. The vials were mixed by vortexing and stored at -70°C.

2.3 Culture media

Unless otherwise stated, all media was sterilised by autoclaving at 15 lbs psi for 15 minutes.

2.3.1 Solid media

Nutrient agar, IsoSensitest (IST) agar and MacConkey agar were all obtained from Oxoid. They were prepared according to the manufacturer's instructions and sterilised as described (section 2.3). The agar was allowed to cool to 50°C before being poured into sterile petri dishes (Sterilin Ltd, Stone, Staffordshire) and allowed to set. The plates were then stored at 4°C.

Table 2.1 Clinical isolates of *Acinetobacter* species and their sources

REFERENCE NUMBER	SOURCE
779,780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 883, 884, 885, 886, 887	Dr. C. Bantar, Dr. J. Smayevsky, Buenos Aires, Argentina
806, 807, 808, 809	Dr. J. Vila, Barcelona, Spain
811, 812, 813, 814, 815 ,816	Dr. J. Ling, Hong Kong
820 ,821, 822, 823, 824, 825, 826, 827, 829, 830, 831, 832, 833, 834, 836, 837, 838, 839, 840, 841, 843, 844, 847, 848, 849, 850,	Professors Z. Gülay & D. Gür, Ankara, Turkey
860, 861, 862, 863, 864, 865, 866, 867, 868, 872, 873	Dr. R. Lin, Singapore

2.3.2 Liquid media

Nutrient broth and IST broth from Oxoid were prepared according to the manufacturer's instructions and sterilised as previously described. Luria Bertani (LB) Broth was prepared by dissolving 10 g of tryptone (Difco Laboratories, Detroit, Michegan), 5 g of yeast extract (Difco), and 1 g of glucose (Sigma Chemicals, Poole, Dorset) in 900 ml of distilled water. The pH was adjusted to pH 7.0 with 1N NaOH (Sigma), the volume was adjusted to 1 litre with distilled water, and then sterilised as previously described.

2.4 Reagents

All reagents were obtained from Sigma Chemicals unless otherwise stated.

Table 2.2 Standard strains and β-lactamase-producing strains

Strain	Characteristics	Source/Reference
E. coli NCTC 10418 *P. aeruginosa* NCTC 10662 *S. aureus* NCTC 6571	sensitive laboratory standards	National Collection of Type Cultures, London
A. baumannii 6B92	bla_{ARI-1}	Paton *et al.*, 1993
S. maltophilia ULA-511	bla_{L1}	Felici *et al.*, 1993
E. coli K-12 J62-2	bla_{TEM-1}	S. G. B. Amyes*
E. coli J53-2	bla_{SHV-1}	S. G. B. Amyes*
E. coli K12	bla_{SHV-3}	S. G. B. Amyes*
E. coli K12	bla_{SHV-5}	S. G. B. Amyes*
E. coli K12	bla_{OXA-2}	S. G. B. Amyes*
P. aeruginosa M18	bla_{IMP}	Dr Y. Arakawa
S. marcescens S6	bla_{Sme-1}	Yang *et al.*, 1990
E. cloacae NOR-1	bla_{NMC-A}	Nordman *et al.*, 1993
Acinetobacter sp. BD413-2	*ura*$^-$, Sm^R, Rif^R	Juni and Janick, 1969
Acinetobacter sp. BD413-2 transconjugant	bla_{ARI-1}	Wendy Scaife, Dundee University

*personal culture collection

2.5 Antimicrobial susceptibility testing

2.5.1 Antimicrobial agents

Table 2.3 lists the antibiotics and their suppliers. All compounds were dissolved in the recommended diluents of the Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy (1991).

Table 2.3 Antimicrobials and suppliers

Antimicrobial	Supplier
Ampicillin, Aztreonam, Benzylpenicillin, Cefotaxime, Cefuroxime, Cephaloridine, Cloxacillin, Oxacillin, Trimethoprim, Norfloxacin	Sigma Chemicals
Ciprofloxacin, Moxifloxacin	Bayer UK
BRL 42715, Clavulanic acid, Nitrocephin	SmithKline Beecham Pharmaceuticals
Ceftazidime, Grepafloxacin	GlaxoWellcome
Imipenem	Merck Sharp & Dohme
Meropenem	Zeneca Pharmaceuticals
Rifampicin	Marion Merrell Ltd
Sparfloxacin	Dainippon, Japan
Tazobactam	Lederle
Trovafloxacin, Sulbactam	Pfizer Inc
Gentamicin	David Bull Laboratories, Warwick

2.5.2 Minimum Inhibitory Concentrations (MICs)

The minimum inhibitory concentrations of antibiotics were determined according to the guidelines of the Working Party on Antibiotic Sensitivity Testing (1991, 1998a). Bacterial strains were grown overnight at 37°C in IST broth and diluted in 0.9% saline to give 10^7 colony-forming units per ml (cfu/ml). A Denley multipoint inoculator (Denley, Surrey) was used to inoculate 1 µl of the diluted suspension onto agar plates containing serially diluted antibiotics to give a final inoculum of 10^4 cfu/spot. The recommended NCTC strains were included as controls (Working Party on Antibiotic Sensitivity Testing, 1991).

MIC values were defined as the lowest concentration of antibiotic that inhibited visible bacterial growth after overnight incubation.

2.5.3 Disc sensitivity testing

This was performed and interpreted by Stoke's method (Holt and Brown, 1989) on IST agar. Antibiotic discs were supplied by Mast Laboratories Ltd, Liverpool.

2.5.4 Dose response curve

Bacterial cultures were challenged with increasing doses of antibiotic and the effect of this on viability was determined. A 10 ml volume of IST broth was inoculated with the strain of interest and incubated overnight at 37°C. The broth was diluted 1:50 in 0.9% saline and incubated with shaking (200 osc/min) at 37°C for 3 hours. A series of 10 ml volumes of IST broth was prepared, each containing a doubling dilution of the antibiotic, to give a range of 1-128 mg/L. An additional broth without antibiotic was used as a control to obtain an initial viable count. Each broth was inoculated with 100 µl of the diluted broth culture, including the antibiotic-free broth, of which 100 µl was removed and inoculated onto a MacConkey agar plate which was incubated overnight at 37°C. The broths were incubated for 3 hours with shaking (200 osc/min) at 37°C after which they were diluted in 0.9% saline and placed on ice. Dilutions were inoculated onto MacConkey agar plates. The plates were incubated overnight at 37°C and the percentage viability determined by relating the viable counts to the initial count, which was taken as 100%.

2.5.5 Determination of combined bacteriostatic action by chequerboard titration

Dilutions of antibiotics were incorporated into IST agar plates to give a series of antibiotic combinations. Final concentrations for imipenem were 4, 8, 12, and 16 mg/L, and for sulbactam were 0.5, 1, 1.5, 2, 12, 18, 24, and 32 mg/L. Plates were

also prepared which contained each antibiotic dilution alone, to verify MIC values of the antibiotics. Representative strains were grown overnight in IST broth at 37°C. The broths were diluted in 0.9% saline and the plates inoculated with the diluted cultures as previously described (section 2.5.2). After overnight incubation, the MIC values of each antibiotic combination were recorded. The combined activity was expressed as the summed fractional inhibitory concentration ($\sum$ FIC) which was calculated as follows:

$$\sum \text{FIC} = \frac{\text{MIC drug A in combination}}{\text{MIC drug A}} + \frac{\text{MIC drug B in combination}}{\text{MIC drug B}}$$

Synergy is indicated by a value of <0.7, addition by a value between 0.71-1.29 and antagonism by a value of >1.3.

2.5.6 Time-kill assays

Representative strains were grown in IST broth overnight at 37°C. The overnight culture was diluted 1:50 in IST broth and incubated for 3 hours at 37°C with shaking (200 osc/min). MacConkey agar plates were inoculated with appropriate dilutions of the log phase culture and incubated overnight at 37°C to obtain the viable count.

A series of tubes were prepared containing IST broth with appropriate antibiotic combinations to which aliquots of each log phase culture were added. The tubes were incubated at 37°C for a period of 5 hours. At 1 hour intervals during this period, aliquots were removed from each tube and diluted in 0.9% saline. Each dilution was used to inoculate MacConkey agar plates which were incubated overnight at 37°C. Viable counts were determined after overnight incubation.

2.6 Preparation of β-lactamases

2.6.1 Small-scale preparation

Bacterial strains were inoculated onto nutrient agar slopes and incubated overnight at 37°C. The slopes were washed with 1 ml of 50 mM sodium phosphate buffer, pH 7.0 and the cell suspensions were transferred into bijou bottles. Cells were subjected to ultrasonication (MSE Soniprep 150, MSE Instruments, Crawley) for 3 x 30 seconds at an amplitude of 8 microns with constant cooling, and with a 1 minute cooling period between each sonication. The cell lysate was cleared by centrifugation (MSE Microcentaur centrifuge) at high speed (13 000 x g) for 10 minutes at 4°C. The cell-free β-lactamase extracts were stored at -20°C until required.

2.6.2 Large-scale preparation

The bacterial strain under investigation was grown in 10 ml of sterile nutrient broth overnight with shaking (200 osc/min) at 37°C. A 1ml volume of the overnight broth was added to 100 ml of pre-warmed nutrient broth in a 250 ml flask and incubated overnight as previously described. Cells were harvested by centrifugation at 6084 x g (GS3 rotor, Sorvall® RC-5B, Du Pont) for 15 minutes at 4°C. The cell pellet was washed with 10 ml of 50 mM sodium phosphate buffer pH 7.0 and the suspension centrifuged (H1000B rotor, Sorvall RT 6000D, Du Pont) at 1521 x g for 15 minutes at 4°C. The pellet was resuspended in 4 ml of buffer and the β-lactamase content released by ultrasonication and stored as previously described (section 2.6.1).

2.6.3 Induction of β-lactamases

The bacterial strain was grown overnight in 10 ml of nutrient broth as described in section 2.6.2. This broth was added to 100 ml of pre-warmed nutrient broth in a 250 ml flask and incubated at 37°C with shaking (200 osc/min) for 90 minutes. At this stage, imipenem was added at ¼ the strain's MIC as a β-lactamase inducer

(Reid, 1986). After incubation for a further 2 hours, the cells were harvested, washed and sonicated as previously described (section 2.6.2).

2.6.4 Preparation of β-lactamases for anion exchange column

The bacterial strain was grown in 10 ml of LB broth overnight with shaking (200 osc/min) at 37°C. The broth was added to 1 L of pre-warmed LB broth in a 2 L flask and incubated overnight as previously described, and the culture was divided into 4 x 250 ml and centrifuged (GS3 rotor, Sorvall® RC-5B) at 6084 x g at 4°C. The cells were washed and resuspended in 2 x 20 ml of 50 mM Tris-HCl buffer pH 8.2, and centrifuged as previously described. The cell pellets were subsequently resuspended in 2 x 5 ml of buffer and subjected to ultrasonication as previously described (section 2.6.1). The cell lysate was centrifuged (70.38 rotor, Sorvall Ultracentrifuge OTD 65B, Du Pont) at 32,000 x g for 1 hour at 4°C, and the supernatant containing β-lactamase was removed and filtered by syringe through a 0.22 μm Durapore membrane filter (13 mm diameter, Millipore). The extract was stored at 4°C until ready to be applied to the column.

2.6.5 Detection of β-lactamase activity by the nitrocephin spot assay

A 30 μl volume of β-lactamase preparation was added to 100 μl of nitrocephin (50 mg/L) in a microtitre plate. The colour change of the solution from yellow to red indicated β-lactamase activity, and the time in seconds for this change to take place was recorded.

2.7 Analytical isoelectric focusing (IEF)

β-lactamases were identified by IEF as described by Matthew *et al.* (1975). The extracts were focused on a horizontal thin layer polyacrylamide gel containing ampholines with a range of pH 3.5-10.

2.7.1 Gel casting

Two glass plates were separated by rubber tubing placed along their perimeters, and clamped together to form a casting chamber into which the gel solution was poured. Prior to assembly, one of the glass plates was coated with a binding solution (0.5% w/v gelatine, 0.5% w/v chromium potassium sulphate dodecahydrate), and dried at 55°C. This promoted adhesion of the gel to the glass. The other plate was siliconised to permit easy separation of the polymerized gel.

Table 2.4 lists the components of the gel in the order they were added. Once the gel solution had been poured between the glass plates, it was left to polymerise for at least 4 hours.

Table 2.4 Composition of an IEF gel

Material	Volume added (ml)	Final concentration
Distilled water	25	-
40% (w/v) ampholines pH 3.5-10	2.0	2% (w/v)
100 g acrylamide plus 2.7 g methylene bisacrylamide (BDH) in 300 ml distilled water	9.0	acrylamide - 75 g/L bisacrylamide - 2 g/L
Riboflavin (20 mg/L)	4.0	2 mg/L
5% (v/v)TEMED*	0.2	0.005% (v/v)

*Tetramethyl-ethylenediamine

2.7.2 Loading IEF gels

The volume (μl) of β-lactamase preparation that was equivalent to its nitrocephin spot test time, was applied to the gel surface close to the anode. A maximum of 15 μl of

preparation was added per lane. β-lactamase preparations of known isoelectric points (pIs) were also applied to the gel in order to quantify the gradient.

2.7.3 Running conditions

Focusing was performed at 4°C, 500V (limiting), 1W (constant) and 20mA (limiting) for 18 hours.

2.7.4 Pre-poured polyacrylamide gels

When a large number of β-lactamase extracts were to be analysed at one time, pre-poured gels (Ampholine® PAGplate, Pharmacia Biotech, Uppsala, Sweden) containing 2% (v/v) of broad range ampholines, pH 3.5-9.5 were used. The gel was electrophoresed on a LKB 2217 Ultraphore Electrophoresis Unit (Pharmacia Biotech) according to the manufacturer's instructions.

2.7.5 Visualisation of β-lactamases after electrophoresis

β-lactamase activity was detected by soaking a piece of filter paper (Whatman® No. 1, Whatman International Ltd, Maidstone) in nitrocephin solution (50 mg/L) and overlaying the surface of the gel. The focused β-lactamases appeared as red bands on a yellow background. Stained gels were photographed with a Polaroid camera using a Tiffen green filter.

2.7.6 Inhibitor overlays

To detect β-lactamase inhibition, filter paper (Whatman® No. 1) was soaked in the required concentration of inhibitor solution and laid over the gel surface. The gel plus filter paper was incubated at 37°C for 5 minutes before the filter paper was removed and the gel stained as described (section 2.7.5).

2.8 Microbiological assays

2.8.1 Method of Paton *et al.*, 1993.

This method as described by Paton *et al* (1993) was employed to detect β-lactamase activity in extracts prepared as described in sections 2.6.1 and 2.6.2. The indicator organism, *S. aureus* NCTC 6571 was grown in IST broth at 37°C overnight and diluted in 0.9% saline to give an optical density of 0.2 at 550nm. The suspension was spread onto an IST agar plate. An antibiotic disc containing the choice antibiotic was placed in the centre of the plate. Filter paper discs containing 10 µl of β-lactamase extract either neat, or diluted 1:2, 1:4, or 1:8 in 50 mM sodium phosphate buffer pH 7.0, were placed at the periphery of the expected zone of inhibition (determined by incubating a plate with indicator organism and antibiotic disc only). After overnight incubation at 37°C, any growth of the indicator organism observed around the filter paper discs was recorded.

2.8.2 Modified microbiological method

In order to quantify the hydrolysis of imipenem by β-lactamase preparations demonstrated by the microbiological assay (section 2.8.1), a modification of this method was developed. β-lactamase preparations were incubated with antibiotic over a period of 1 ½ hours. The concentration of imipenem remaining at several time points within this period was subsequently determined from a standard graph from which the rate of imipenem hydrolysis could be determined.

2.8.2.1 Preparation of standard curve of imipenem concentration against diameter of inhibition zones

Molten IST agar was pipetted into 2 large (140 mm diameter) petri dishes (60 ml per petri) and allowed to set. A 10 ml volume of IST broth was inoculated with a standard strain of *S. aureus* (NCTC 6571) and incubated overnight at 37°C. The

following day, the broth was standardised with 0.9% saline to give an optical density of 0.2 at 550nm. The IST agar plates were subsequently inoculated with the standardised culture and the surfaces were allowed to dry at room temperature. Using a glass tube that had been sterilised in absolute alcohol, 3 holes were made in the agar of each plate, and the cut agar was removed. A series of doubling dilutions of imipenem was prepared (range 10^{-5} M to 0.312 x 10^{-6} M) in 50 mM sodium phosphate buffer pH 7.5. Aliquots of 20 µl of each dilution were then added to the appropriately labelled wells and the plates incubated overnight at 37°C. The following day, the zones of inhibition were measured and recorded. The procedure was repeated twice and the mean value of each zone size was plotted against imipenem concentrations on a semi-logarithmic scale.

2.8.2.2 Determination of the rate of hydrolysis of imipenem by crude β-lactamase extracts

A 250 µl volume of crude β-lactamase extract was placed in a sterile Eppendorf tube to which 25 µl of imipenem 10^{-4} M was added to give a final antibiotic concentration of 10^{-5} M. After mixing, a 25 µl volume was immediately transferred to a fresh Eppendorf and incubated at 70°C for 4 minutes to inactivate the β-lactamase. (The time period and temperature for complete enzyme inactivation without degradation of antibiotic were determined by incubating enzyme/antibiotic solutions at varying temperatures over a period of 15 minutes). The sample was subsequently placed on ice. The remaining enzyme/antibiotic solution was incubated at 37°C and after 15 minutes, another 25 µl aliquot was removed and incubated at 70°C for 4 minutes before being placed on ice. This procedure was repeated at 15 minute intervals over a 1 ½ hour period. At the end of this time, the wells in the inoculated IST agar plates were filled with 20 µl of the appropriate inactivated enzyme/antibiotic solutions. Following overnight incubation at 37°C, the zones of inhibition were measured and recorded. The relative concentrations of antibiotic remaining after each 15 minute period during the 1 ½ hours were determined by plotting the zone sizes on the standard graph. The concentrations obtained from this graph were subsequently

plotted against time. The initial rate of hydrolysis was measured as nmoles of imipenem hydrolysed/min. Specific activities were determined by calculating the protein concentration of β-lactamase extract by the method of Waddell (1956) as described in section 2.12.2.

2.8.2.3 Determination of antibiotic instability

To confirm that the loss of antibiotic activity was due solely to hydrolysis by the β-lactamase extract rather than to instability of the substrate, aliquots of a solution of imipenem (10^{-5} M) were incubated under the same conditions as the test solution and added to the wells in appropriately labelled IST agar plates which had been seeded with the *S. aureus* standard strain. The plates were incubated overnight at 37°C, after which time the zone sizes were measured and any loss of activity as a result of substrate instability was taken into account when calculating the rate of hydrolysis.

2.9 Conjugation studies

For both methods employed, donor (test strain) and recipient (*Acinetobacter* sp. BD413-2) strains were grown independently in 4.5 ml of nutrient broth and incubated overnight statically at optimum temperature. Matings were performed at 25°C and 37°C. Controls of each strain were prepared by inoculating 0.1 ml volumes of each broth onto separate nutrient agar plates containing 4 mg/L of imipenem (to select donor colonies) and 50 mg/L of rifampicin (recipient-selective). In addition, a total viable count of the donor strain was performed by the technique of Miles and Misra (1938). Both control plates and viable count plates were incubated at the same temperatures as the mating mixtures.

2.9.1 Broth matings

Broth matings were performed as described by Amyes and Gould (1984). A 0.1 ml volume of donor broth and 1.0 ml volume of recipient broth were added to 4.5 ml of

pre-warmed nutrient broth, and incubated statically overnight at the designated temperature. The mating mixture was vortexed to separate the conjugating pairs and serially diluted in 0.9% saline. A 100 µl aliquot of each dilution was spread onto antibiotic-containing nutrient agar plates selective for transconjugants (4 mg/L imipenem and 50 mg/L rifampicin). The plates were incubated at the optimum temperature overnight or until colonies were visible.

2.9.2 Membrane filter matings

Membrane filter matings were performed by the method of Willets (1988). Donor and recipient strains were grown in 10 ml of nutrient broth overnight at the appropriate temperature. After incubation, 0.2 ml of donor culture and 1.8 ml of recipient culture were mixed together. The mixture was filtered through a 0.2 µm pore cellulose acetate filter (Whatman), using a sterile 5 ml disposable tip and pipette. Sterile forceps were used to place the filter onto a prewarmed nutrient agar plate (cells uppermost), which was incubated at the designated temperature for 2 hours. The filter was immersed in 2 ml of nutrient both which was subsequently vortexed to resuspend the cells. Appropriate dilutions of the suspension were plated onto selective plates and incubated for a suitable duration at the optimum temperature.

2.10 Plasmid elimination by ethidium bromide

To determine whether the genes of imipenem-hydrolysing β-lactamases were plasmid-encoded, strains were treated with ethidium bromide, which preferentially inhibits plasmid synthesis (Bouanchaud *et al.*, 1969). The bacterial culture was grown overnight at 37°C in 10 ml of nutrient broth. The culture was diluted 1:100 in 0.9% saline. Tubes of nutrient broth containing serial dilutions of ethidium bromide were prepared. The range of dilutions was dependant on the tolerance of the strain to ethidium bromide. If this was not known, the MIC was determined (section 2.5.2), and the range was altered accordingly to include this value. The tubes were each inoculated with 20 µl of diluted culture and incubated overnight at 37°C.

The tube that contained the highest concentration of ethidium bromide and demonstrated visible bacterial growth was diluted 1:100 in 0.9% saline (to obtain single colonies), and 100 µl of this suspension was inoculated onto 2 or 3 nutrient agar plates. The plates were incubated overnight at 37°C. After incubation, a series of 4.5 ml volumes of 0.9% saline were inoculated with between 40 and 50 distinct colonies from the plates. Nutrient agar plates containing a sub-inhibitory concentration of imipenem were inoculated with 10 µl of each saline suspension. Control plates (no antibiotic) were also inoculated with the same volume. Following overnight incubation at 37°C, the control plates were checked for bacterial growth. The corresponding antibiotic plates were subsequently checked and those that showed no visible growth were recorded. Loss of imipenem resistance was verified by determining the MIC values (section 2.5.2) for colonies that had failed to grow on the imipenem-containing plates. β-lactamase extracts were prepared from colonies that demonstrated a reduction in MIC value, and analysed by IEF (section 2.7).

2.11 Purification of β-lactamases by anion-exchange and gel filtration by Fast Protein Liquid Chromatography (FPLC®) system

2.11.1 Partial purification of β-lactamases by MonoQ HR 5/5 anion-exchange

Crude β-lactamase extract was prepared as described in section 2.6.4 and a 8 ml volume of this applied to a MonoQ anion-exchange column employing the FPLC® system (Pharmacia). The column was run at a flow rate of 0.5 ml/min, and protein was eluted in a 1 mM NaCl gradient (prepared in 50 mM Tris-HCl pH 8.2). Fractions were collected in 1 ml volumes using an LKB 2070 Ultrorac II fraction collector (Pharmacia), and were assayed with nitrocephin (section 2.6.5) to determine β-lactamase activity. The presence of specific β-lactamases in positive fractions was determined by IEF. Fractions containing the β-lactamase of interest were pooled and applied to a gel filtration column.

2.11.2 β-lactamase purification by Superdex 75 Hiload 26/60™ gel filtration

A 7 ml volume of pooled fractions collected from the anion-exchange column was applied to a Pharmacia FPLC Superdex 75 Hiload™ 26/60 gel filtration column (capacity volume of approximately 80 ml). The column was run at a flow rate of 1.5 ml/min, and fractions were collected in 3 ml volumes which were subsequently assayed for β-lactamase activity as previously described. Isoelectric focusing was performed on positive fractions to determine whether separation of β-lactamases had been successful.

2.11.3 Concentration of purified β-lactamases

Fractions collected from the gel filtration column that contained the β-lactamase of interest were pooled together and concentrated using a VectaSpin Micro centrifuge tube filter (Whatman) according to the manufacturer's instructions.

2.12 β-lactamase assays

2.12.1 Substrate hydrolysis assays

Spectrophotometric analysis of β-lactamase hydrolytic activity of a range of β-lactam antibiotics was performed using a double beam UV/V Lambda 2 spectrophotometer (Perkin-Elmer). Assays were performed using 10mm path length cuvettes (Hellma) in 50 mM sodium phosphate buffer pH 7.0 at 37°C for a period of 5 minutes at the wavelength (λ) of maximal absorbance for each β-lactam (Table 2.5).

All substrates were freshly prepared prior to use. Penicillins and carbapenems were assayed at 10^{-3} M, and cephalosporins and nitrocephin at 10^{-4} M (final concentrations). Enzyme samples were kept on ice until ready to be used. Only the initial linear phase of the reaction was used to calculate the change in absorbance rate,

and all rates were related back to that obtained for a standard substrate (ampicillin), the value of which was set at 100.

Table 2.5 Optimal wavelengths for the measurement of β-lactam hydrolysis

Antibiotic	Wavelength (λ_{max})
Ampicillin	238nm
Cefuroxime	260nm
Cefotaxime	265nm
Ceftazidime	260nm
Cephaloridine	255nm
Imipenem	299nm
Meropenem	300nm
Nitrocephin	384nm
Oxacillin	237nm
Cloxacillin	237nm

β-lactamase activity was calculated as the amount of substrate hydrolysed (μmoles) per minute per ml of enzyme sample by the following formula as described by Payne and Farmer (1998).

$$\frac{\Delta Abs\ min^{-1} \times V_r/1000 \times 1/V_e}{\Delta\sum \times 10^{-6}}$$

$\Delta Abs\ min^{-1}$ = rate of absorbance change

$\Delta\sum$ = molar extinction coefficient of hydrolysis*

V_r = volume of the reaction (ml)

V_e = volume of enzyme added

*The molar extinction coefficient was determined by measuring the absorbance of a known concentration of β-lactam at its optimal wavelength (Table 2.5).

These values were inserted into Beer Lambert's equation to obtain the molar extinction coefficient:

$$A = e\ c\ l$$

where A = absorbance, e = molar extinction coefficient, c = concentration of β-lactam measured, l = cuvette path length (cm)

2.12.2 Specific activities

The specific β-lactamase activity was calculated by determining the protein concentration of β-lactamase sample (mg/ml) by the method of Waddell (1956). The sample was diluted 1:1000 in 50 mM phosphate buffer pH 7.0 and the absorbance measured at 215 nm and 225 nm. The difference in absorbance between the two measurements was multiplied by the correction factor (168) determined by a calibration curve with this spectrophotometer. Specific activity was expressed as nmoles of substrate hydrolysed min^{-1} mg^{-1} protein.

2.12.3 Determination of V_{max} and K_m values

Values were determined by linear regression analysis of Lineweaver-Burk plots of the initial velocity data obtained at different substrate concentrations (Lineweaver and Burk, 1934). The assayed range of penicillins and carbapenems was 0.25-4 mM, and 0.025-0.4 mM for cephalosporins. V_{max} is defined as the velocity of reaction produced by a given enzyme concentration in vast excess of substrate. K_m is the Michaelis constant for a substrate, and is the substrate concentration that gives half the maximum velocity. Values were obtained by plotting the reciprocal of substrate concentrations against the reciprocal of the rate of hydrolysis.

2.12.4 Inhibition assays

The ID_{50} value is defined as the concentration of inhibitor required to reduce the hydrolytic activity of the β-lactamase by 50%. The β-lactamase was incubated with a

range of inhibitor concentrations for 10 minutes at 37°C. A reporter substrate (nitrocephin at 100 μM) was subsequently added and the initial rate of hydrolysis determined as previously described. The ID_{50} values were determined by plotting the percentage inhibition against $\log_{10}$ concentration of inhibitor. The concentration of inhibitor that gave 50% inhibition was calculated from this graph.

2.13 Determination of β-lactamase molecular mass (M_r) by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were mixed with an equal volume of SDS-PAGE sample buffer (stock = 4 ml distilled water, 1 ml 0.5 M Tris-HCl, pH 6.8, 800 μl glycerol, 1.6 ml 10% (w/v) SDS, 400 μl β-mercaptoethanol, 200 μl 1% bromophenol blue), heated at 100°C for 5 minutes, and kept on ice until required for gel loading. A 35 μl volume of each sample was loaded into the wells of a 4-15% linear gradient Tris-HCl Ready-Gel (Bio Rad) in duplicate so that each half of the gel contained each sample. Low range molecular weight markers (Bio Rad) were added to a well on one side of the gel only. The gel was inserted into a minigel electrophoresis apparatus (Miniprotean® II, Bio Rad) and electrophoresis was carried out at 200 V for approximately 30 minutes or until the dye in the sample buffer had reached the end of the gel.

The gel was removed from its casing and cut in two using a scalpel. One gel half was immersed in the non-ionic detergent, Triton X-100 (1% v/v in 50 mM Tris-HCl pH 7.5) for 4 hours at 37°C to renature the β-lactamases. The gel was then immersed in nitrocephin (50 mg/L) until bands were visualised. The positions of the bands (from the bottom of each well) and the gel length were measured (mm) and recorded.

The other gel half containing the molecular weight standards was stained in Coomassie Blue (40% v/v methanol, 10% v/v acetic acid, 0.25% w/v Coomassie blue R-250) with gentle shaking for ½ to one hour. The gel was transferred to a destaining solution (40% v/v methanol, 10% v/v acetic acid in distilled water) and destained with

gentle shaking for approximately 4 hours, or until the background became clear. Destaining solution was replaced with fresh solution several times during this period. As this procedure results in shrinkage, the gel was subsequently immersed in 20% (v/v) glycerol until it resumed its original length.

2.14 N-terminal amino-acid sequencing of purified β-lactamase

All electrophoresis and electroblotting reagents were prepared in ultra pure pyrogen-free water (MilliQPF, Millipore). Stains were filtered (Whatman® No. 1 filter paper) before use.

2.14.1 Native-PAGE of purified β-lactamase

A 10% polyacrylamide gel was prepared as follows:

15 ml of acrylamide (Phigel 2 29:1 acrylamide:bisacrylamide, Fisher Scientific)
25 ml MilliQ water
20 ml 0.25 mM tris/glycine pH 8.8
80µl TEMED

A small amount of ammonium persulphate was added with the tip of a spatula to the above solution and mixed gently, avoiding the addition of air which affects the gel matrix. The solution was poured into the preassembled gel apparatus (Protean II xi Cell, Bio Rad). A 10 well comb was inserted, and the gel allowed to polymerise for at least 45 minutes.

Samples were prepared by adding 8 µl each of bromophenol blue and 25% (w/v) sucrose to 65 µl of purified β-lactamase and mixing gently.

The top chamber of the electrophoresis unit was filled with 0.1 M Tris/glycine pH 8.8 containing 0.002 M thioglycolic acid, and the bottom chamber with 0.1 M

Tris/glycine pH 8.8. Samples were loaded and electrophoresis was carried out at 250V for approximately 7 hours, or until adequate separation of protein bands had been achieved.

After the gel had been removed from the electrophoresis apparatus, it was immersed in nitrocephin (50 mg/L) and the positions of β-lactamase bands were measured as previously described (section 2.13). The gel was subsequently stained with Coomassie blue (0.025% w/v Coomassie blue R-250, 25% v/v isopropanol, 10% v/v acetic acid) overnight at 37°C, followed by destaining (16.5% v/v methanol, 5% v/v acetic acid) with gently agitation until a relatively clear background was obtained. The stained gel was photographed and the protein band(s) of interest (that corresponded to those visualised by the activity stain) were noted.

2.14.2 Protein immobilisation

Proteins were transferred from the polyacrylamide gel onto a PVDF membrane (ProBlot™, Applied Biosystems).

A piece of membrane was cut to the size of the gel and immersed in 100% methanol for a few seconds. The membrane and the gel were subsequently equilibrated by placing them in a glass dish containing electrophoresis buffer (25 mM Trizma base, 190 mM glycine, 20% v/v methanol) for 10 minutes. A Trans-Blot® Electrophoretic Transfer Cell (Bio Rad) was assembled as described by the manufacturer. Electroblotting was carried out at 30 V overnight, after which the apparatus was disassembled and the membrane removed from the transblotting sandwich.

2.14.3 Protein detection

The membrane was stained (0.1% w/v Coomassie blue R-250, 50% v/v methanol, 1% v/v acetic acid in MilliQ water, filtered) for 30 minutes, followed by 2 x 5 minutes destaining (50% v/v methanol) and 2 x 5 minutes washing in MilliQ water. The membrane was placed between 2 sheets of filter paper (Whatman® No. 1) and allowed to dry overnight.

2.14.4 N-terminal amino-acid sequencing

N-terminal amino-acid sequencing of the protein bands of interest was performed by Edman degradation (Hayes *et al.*, 1989) on an Applied Biosystems Procise Sequencer.

2.15 Protein separation by Reversed-Phase High-Performance Liquid Chromatography (RPHPLC)

The concentrated pooled fractions from the Superdex 75 column were subjected to reversed-phase HPLC in an attempt to separate their protein content. A 200 µl aliquot of the fractions was passed through a ABI 130 A Microbore HPLC system. Solvents and running conditions used were as follows:

Cartridge: Aquapore RP300 C8 (7 µm particle size, 30 mm x 2.1 mm)
Solvent A: 0.1% Trifluoroacetic acid (TFA) in 0.1% TFA in water
Solvent B: 0.08% TFA in 70% acetonitrile

Running conditions - Gradient: linear 10-20% solvent B over 45 minutes
Flow rate: 200 µl/min
Chart speed: 2 mm/min
Wavelength: 220 nm

Protein peaks were collected manually in sterile Eppendorfs, and centrifuged under vacuum with cooling for approximately 4 hours, or until most of the solvent had evaporated.

2.15.1 Native-PAGE of protein peaks collected from RPHPLC

Samples were diluted 1:3 in sample buffer and loaded on a 15% single gradient Tris-HCl Ready Gel (Bio Rad). Electrophoresis was carried out as previously described (section 2.13) and the gels were stained by silver stain (Pharmacia Biotech) according to the manufacturer's instructions.

2.16 Preparation of DNA

2.16.1 Plasmid DNA extraction by alkaline lysis

The method used was a modification of the method described by Birnboim and Doly (1979).

The bacterial strain was grown in 2 x 10 ml LB broths overnight at 37°C with agitation (200 osc/min). The culture was transferred into 1.5 ml Eppendorfs and centrifuged at 13 000 x g for 5 minutes. The supernatant from each Eppendorf was removed by vacuum and discarded. The pellets were resuspended in 100 µl of Solution I (25 mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM Na_2EDTA) by vortexing to completely resuspend the cells. A 100 µl volume of freshly prepared lysozyme (4 mg/ml in Solution 1) was added to each Eppendorf and mixed by inverting, before placing on ice for 5 minutes. A 400 µl volume of freshly prepared Solution II (0.2 M NaOH, 1% SDS w/v) was subsequently added to each tube. The contents were mixed by inverting the Eppendorfs until the solution became translucent. Following a period of 5 minutes on ice, 300 µl of Solution III (3 M sodium acetate, pH 4.8) was added to each Eppendorf, the contents were mixed by inversion and the Eppendorfs returned to ice for a further 20 minutes. The resulting precipitates were pelleted by centrifugation at 13 000 x g for 15 minutes, after which

the supernatant from each tube was pooled and transferred in 750 µl aliquots into fresh Eppendorf tubes.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and the contents inverted for 2 minutes before being centrifuged at 13 000 x g for 5 minutes. The clear top layer above the interface was removed from each tube, pooled together and aliquoted in 750 µl amounts into fresh Eppendorf tubes. Ice-cold propan-2-ol (450 µl) was subsequently added to each tube, the contents mixed by inversion and stored on ice for 30 minutes, after which they were centrifuged at 13 000 x g for 10 minutes. The propanol was removed by vacuum and 1 ml of 70% (v/v) ethanol (room temperature) was added to each Eppendorf and the contents mixed by inverting several times. The tubes were centrifuged at 13 000 x g for 5 minutes and the supernatant removed by a vacuum pump and discarded. The tubes were re-centrifuged at the same speed for a few seconds and any remaining ethanol removed again by a vacuum pump. The DNA pellets were allowed to dry at room temperature for at least 45 minutes. TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA) (50 µl) was added to one of the Eppendorfs to resuspend the DNA. The contents of this tube were subsequently added to the second Eppendorf, and the resuspension procedure repeated until all the DNA had been pooled together. The DNA was stored at -20°C.

2.16.2 Extraction of chromosomal DNA

Genomic DNA was extracted using the Puregene® DNA Isolation Kit (Gentra Systems) according to the manufacturer's instructions.

2.16.3 Rapid estimation of DNA yield

The concentration of DNA prepared as described in sections 2.16.1 and 2.16.2 was estimated by the ethidium bromide dot quantitation method as described in Unit 2.6 in Current Protocols in Molecular Biology (Moore *et al.*, 1995).

2.17 Agarose gel electrophoresis

Electrophoresis was performed for the analysis of DNA (sections 2.16.1 and 2.16.2), and to identify DNA fragments after enzyme restriction. This was usually performed in 1% agarose gels.

2.17.1 Preparation of agarose gel

Electrophoresis grade agarose (Gibco BRL, Life Technologies, Paisley) was added to 1 x TAE buffer (10 x TAE stock: 400 mM tris-acetic acid pH 8.0, 20 mM Na_2EDTA) to give the required gel concentration (typically 1-2%). The agarose was dissolved completely by heating in a microwave in 20 second bursts with gentle swirling between heating. The agarose was allowed to cool to approximately 55°C before carefully pouring into the gel casting tray into which a gel comb had been inserted. The gel was allowed to set completely before the comb was removed.

2.17.2 Sample loading and running conditions

The agarose gel was submerged in a horizontal electrophoresis tank (Mini-Protean® II Sub-Cell, Bio Rad) which had been filled with 1 x TAE buffer so that the gel was covered to a depth of about 1 mm. DNA samples (typically 10 µl) were mixed with 3 µl of 10 x loading buffer (10 x stock: 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 40% w/v sucrose), and loaded into the wells with a pipette. DNA molecular weight standards (either λ *Hin*d III fragments or 100 bp ladder, both from Gibco BRL), were also loaded at a concentration of approximately 0.5 µg/lane.

Electrophoresis was performed at 100 V (constant). The power was turned off when the bromophenol blue tracking dye in the loading buffer (which migrates at approximately the same speed as DNA of size 0.5 kb) had migrated approximately ¾ the length of the gel.

2.17.3 Staining and visualisation of DNA

Gels were immersed in ethidium bromide solution (0.5 mg/L) for between 10-30 minutes depending on the concentration of DNA being analysed. If necessary, destaining was carried out with gentle agitation in distilled water to remove excess ethidium bromide and reduce background fluorescence. DNA was visualised by placing the gels on a UV transilluminator (UV Products) and photographed.

2.18 Restriction endonuclease digestion of DNA

The following components were pipetted into a clean Eppendorf tube:

x µl DNA (0.1-4 µg DNA in distilled water or TE buffer)
2 µl 10x restriction endonuclease buffer (supplied with enzyme)
1-5 U restriction enzyme
x µl sterile distilled water (to give a total reaction volume of 20 µl)

The contents were mixed by pulsing in a microcentrifuge for a few seconds, and incubated in a water bath at the recommended temperature for the enzyme used (typically 37°C) for a minimum of 2 hours. The reaction was stopped by the addition of 0.5 µl of 0.5 M Na_2EDTA. DNA fragments were electrophoresed and visualised as previously described (section 2.17).

2.19 Transformation of plasmid DNA into *Acinetobacter* sp. BD413-2

Transformation was carried out by the method of Cruze *et al* (1979). The naturally competent strain, *Acinetobacter* sp. BD313-2 was grown overnight in 10 ml of LB broth at 28°C with agitation (200 osc/min). The overnight broth (20 µl) was inoculated into 20 ml of pre-warmed LB broth held in a 100 ml conical flask, and

incubated at 28°C with agitation until the OD_{550} was between 0.3 and 0.4 (indicative of mid-exponential growth phase). This culture (200 µl) was added to a sterile Eppendorf to which the plasmid DNA had been added (approximately 50 ng in 50 µl of sterile distilled water). The tube was incubated for 1 hour at 28°C with vigorous shaking (300 osc/min), after which transformants were selected by inoculating 100 µl of appropriately diluted culture onto selective media (32 mg/L rifampicin and 1 mg/L imipenem). The plates were incubated at 28°C until colonies were visible.

2.20 DNA amplification by the Polymerase Chain Reaction (PCR)

2.20.1 Reaction volumes

PCR reactions were performed in total volumes of 100 µl. Approximately 25 ng of template DNA was used per reaction. Table 2.6 lists the reaction components which were added in that order to the DNA, in a sterile 0.5 ml thin-walled PCR tube (Advanced Biotechnologies). Negative controls were also included, which comprised a) all components minus template DNA, and b) all components minus primers. Once all the components had been added to the tubes, the contents were mixed by pulsing in a microcentrifuge for a few seconds and overlaid with 50 µl of sterile mineral oil to prevent evaporation during the reaction.

2.20.2 Primer design

Primers were either designed with Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), or the sequences were taken from previously published work. All primers were synthesized by Oswell DNA Services (Southampton University, Southampton). Table 2.7 lists primers used for PCR amplifications.

2.20.3 PCR cycling parameters

PCR tubes containing the PCR reaction mixtures were placed in a Cyclogene Thermal Cycler (Techne Ltd, Cambridge). The specific cycling parameters are given in Table 2.8. Primer annealing and extension temperatures varied depending on the GC content and length of the primers.

Table 2.6 PCR reaction components

Component	Stock concentration	Volume added (µl)	Final concentration
10 x Taq Buffer*	100 mM Tris-HCl/ 500 mM KCl	10	10 mM /50 mM
$MgCl_2$*	25 mM	10	2.5mM
dNTPs**	2 mM	10	0.2 mM
Primer 1	10 pmol/µl	1	10 pmol
Primer 2	10 pmol/µl	1	10 pmol
Sterile water			make volume up to 100 µl
Taq DNA polymerase	5 U/µl	5 µl of 1:10 dilution	2.5 U

*Supplied by Promega UK; **2 mM 4dNTP stock - 2 mM of each dNTP (Boehringer Mannheim, Sussex) in TE buffer pH 7.5. Stored at -20°C in 0.5 ml aliquots

2.20.4 Visualisation of PCR products

PCR product was carefully removed from the tube with a pipette inserted through the oil layer. DNA was electrophoresed on a 1% agarose gel and visualised as previously described (sections 2.17.2 and 2.17.3).

Table 2.7 Oligonucleotide primers used for PCR amplifications

Primer pair	Sequence (5' - 3')	Reference
SHV-5'	CGC CGG GTT ATT CTT ATT TGT CGC	Nüesch-Inderbinen *et al.*, 1996
SHV-3'	TCT TTC CGA TGC CGC CGC CAG TCA	
OXA-A	CGA TAG TTG TGG CAG ACG AA	This work
OXA-B	CAC TCA ACC CAT CCT ACC CA	
Helen 3	CCT CAG GTG TGC TGG TTA TTC	Supplied by H. Donald Dundee University
Helen 4	CCC AAC CAG TCT TTC CAA AA	
GyrA1	AAA TCT GCC CGT GTC GTT GGT	Vila *et al.*, 1995
GyrA2	GCC ATA CCT ACG GCG ATA CC	
ParC1	AAA AAT CAG CGC GTA AGT G	Supplied by P. Higgins Edinburgh University
ParC2	CGA GAG TTT GGC TTC GGT AT	
IntA	GTC AAG GTT CTG GAC CAG TTG C	Supplied by H.-K Young Dundee University
IntB	ATC ATC GTC GTA GAG ACG TCG G	
Cass1	TGA TCC GCA TGC CCG TTC CAT ACA G	Supplied by H.-K Young Dundee University
Cass2	GGC AAG CTT AGT AAA GCC CTC GCT AG	

Table 2.8 Primer-specific cycling parameters

Primer pair	DNA denaturation: primer annealing: extension	Final extension cycle
SHV-5' & SHV-3'	94°C – 5 min 1 cycle Followed by: 95°C - 30 sec 68°C – 30 sec } 30 cycles 72°C – 50 sec	72°C – 5 min
OXA-A & OXA-B	94°C – 1 min 60°C – 1 min } 30 cycles 72°C – 3 min	72°C – 5 min
Helen 3 & Helen 4	95°C – 5 min 60°C – 30 sec } 1 cycle 72°C – 1 min Followed by: 95°C – 15 sec 60°C – 30 sec } 30 cycles 72°C – 1 min	72°C – 5 min
GyrA1 & GyrA2	96°C - 30 sec 55°C - 30 sec } 30 cycles 72°C - 2 min	72°C - 5 min
ParC1 & ParC2	96°C - 30 sec 61°C - 30 sec } 30 cycles 72°C - 2 min	72°C - 5 min
IntA and IntB Cass1 and Cass2	94°C - 1 min 55°C - 1 min } 30 cycles 72°C - 3 min	72°C - 10 min

2.20.5 Purification of PCR products

Purification was performed using the QIAquick PCR Purification Kit (Qiagen, West Sussex) according to the manufacturer's instructions.

2.20.6 Sequencing of PCR products

Sequencing of purified PCR products was performed by the dideoxy method on an ABI Prism automated sequencer (Perkin-Elmer).

2.21 DNA:DNA hybridisation

2.21.1 DNA dot blotting

A piece of positively charged nylon membrane (Hybond™-N^+, Amersham Life Science) was cut to an appropriate size and a grid of 0.5 cm x 0.5 cm squares marked on the surface with a blunt pencil. The membrane was submerged in distilled water for 10 minutes. Sample DNA was pipetted into sterile eppendorf tubes to which 1 M NaOH and 200 mM EDTA, pH 8.2 were added to give a final concentration of 0.4 M NaOH/10 mM EDTA. The samples were boiled at 100°C for 10 minutes to denature the DNA, after which they were placed on ice. The wetted membrane was placed over the top of a petri dish so that most of the membrane surface was freely suspended. The DNA samples were centrifuged for 5 seconds and pipetted onto the grid in 3 µl volumes which were allowed to dry before repeating the process. A total of 9 µl of each DNA sample was applied to the membrane. The top right corner of the membrane was cut to mark the orientation of the grid. The membrane was then rinsed briefly in 2 x SSC (1:10 dilution of 20 x stock: 3 M NaCl, 0.3 M Na_3citrate.$2H_2O$, pH adjusted to 7.0 with 1 M HCl) and allowed to air dry.

2.21.2 Southern blotting using an alkali buffer

Restricted DNA was transferred from agarose gels and covalently linked to a Hybond™ -N^+ membrane by an alkaline transfer buffer as described in Unit 2.9 of Current Protocols in Molecular Biology (Brown, 1995).

DNA samples were first restricted with an appropriate restriction endonuclease as previously described (section 2.18), and subjected to electrophoresis on a 1% agarose gel (section 2.17). Transfer of restricted DNA was carried out for a minimum of 2 hours with 0.4 M NaOH as the transfer solution. If dot blotted DNA was to be incorporated onto the same membrane, this was carried out after the membrane had been recovered from the alkaline transfer. The membrane was subsequently rinsed in 2x SSC and placed between 2 pieces of 3MM filter paper (Whatman®) to air dry.

2.21.3 Labelling of DNA probes

Table 2.9 lists the probes used in this study. PCR-amplified DNA was purified as described in section 2.20.5, and the DNA concentration estimated as described in section 2.16.3. An aliquot was diluted to approximately 25 mg/L and denatured by boiling at 100°C for 5 minutes, then chilled on ice. The DNA fragments (50 ng of DNA) were labelled with fluorescein-11-dUTP by the ECL Random Prime Labelling System (Amersham Life Science) and stored at -20°C until required.

Oligonucleotides were labelled at their 3'-end with FI-dUTP by the ECL 3'-Oligolabelling System (Amersham Life Science). Approximately 100 x 10^{-12} moles of oligonucleotide was labelled per reaction.

Table 2.9 Labelled probes used for hybridisation experiments

Probe	Gene/sequence derived from
Gene probes	
ARI-1 intragenic	bla_{ARI-1}
SHV	bla_{SHV-1}
Oligonucleotide probes	
Helen 2	bla_{ARI-1} (TGG AGA ACC AGA AAA CGG AT)
ARI-NB degenerate	Conserved sequence of bla_{OXA} genes (YTC IAC CCA ICC IAC CCA CC)
ARI-2N degenerate	N-terminal sequence of ARI-2 protein (GIA ARA ARA AYA CIG CIC CIG CIG)

2.21.4 Hybridisation procedure

Hybridisations with labelled probes were performed using either the ECL Random Prime Detection System or the ECL 3'-Oligolabelling and Detection System depending on the probe type. The reaction stages requiring temperatures other than room temperature were performed in a hybridisation incubator (Techne Hybridiser HB-1D, Techne Ltd, Cambridge). For room temperature stages, membranes were transferred to plastic boxes which were gently agitated (Mk V Orbital Shaker, LH Engineering). A 20 ml volume of hybridisation buffer was used per membrane and labelled probes were added at a concentration of approximately 20 ng/ml for gene probes, and 10 ng/ml for oligonucleotide probes. Degenerate oligonucleotide probes were used at a higher concentration of approximately 20 ng/ml.

2.21.5 Post-hybridisation washes

The following stringency washes (typically in 100 ml volumes) were carried out for hybridisations with gene probes:

Low stringency -	1 x 15 minutes at 50°C with 1 x SSC, 0.1% SDS
Medium stringency -	1 x 15 minutes at 60°C with 1 x SSC, 0.1% SDS
High stringency -	1 x 15 minutes at 60°C with 1 x SSC, 0.1% SDS followed by 1 x 15 minutes at 60°C with 0.5% SSC, 0.1% SDS

Oligonucleotide probes:

Low stringency -	2 x 5 minutes at room temperature with 5 x SSC, 0.1% SDS
High stringency -	2 x 5 minutes at room temperature with 5 x SSC, 0.1% SDS followed by 1 x 15 minutes at 42°C with 1 x SSC, 0.1% SDS

2.21.6 Signal detection

Hybridisation signals were generated and detected as described in the appropriate detection systems. Autoradiography film (Hyperfilm™ -ECL, Amersham Life Science) was exposed initially for 30 min, and extended up to 5 hours depending on the strength of the signal after the initial period.

2.21.7 Reprobing blots

Membranes were wrapped (wet) in SaranWrap and stored at -20°C after ECL detection, making sure that they did not dry out. When reprobing was required, membranes were rinsed in 5 x SSC for 5 minutes before a solution of 0.1% (w/v) SDS

(which had been brought to the boil) at approximately 5 ml/cm^2 membrane was poured over them. The blots were left at room temperature for 2 hours and rinsed in 5 x SSC for 5 minutes. If reprobing was not to be carried out immediately, the membranes were wrapped wet in SaranWrap and stored at 4°C.

2.22 Analysis of Restriction Fragment Length Polymorphism (RFLP) patterns by Pulsed-Field Gel Electrophoresis (PFGE)

Restricted genomic DNA fragments were separated on a Contour-clamped Homogenous Electric Field (CHEF) electrophoresis system (CHEF-II, Bio Rad) according to the manufacturer's instructions.

2.22.1 Preparation of unsheared genomic DNA in plugs

Strains were inoculated into 10 ml of nutrient broth containing 0.5% yeast extract (Oxoid) and incubated at 37°C overnight with agitation (200 osc/min). The cells were harvested by centrifugation at 1521 x g (H 1000B rotor, Sorvall RT 6000D, Sorvall) for 10 minutes at 4°C. After the supernatant had been discarded, the cell density of each broth was standardised by resuspension in SE Buffer (100 mM NaCl, 25 mM Na_2EDTA, pH 7.5) to give a value of 1.0 OD at 590nm (Lambda 2 spectrophotometer, Perkin-Elmer).

Low melting agarose (Bio Rad) was added to SE buffer (a volume of 500 µl of buffer per strain) to give a final agarose concentration of 2%. The solution was boiled at 100°C for 30 minutes and allowed to cool to 55°C. The standardised cultures (500 µl) were pipetted into sterile eppendorfs, and 500 µl of the molten agarose was subsequently added to each one. The tubes were mixed well by inverting several times and kept molten at 55°C. Plug moulds (Bio Rad) were wiped with ethanol and sealed with tape at the bottom. Each culture/agarose mixture was carefully pipetted into each mould to give a total of 5 plugs per strain. The moulds were then allowed to set at 4°C for approximately 15 minutes, after which time the tape was removed

from the bottom and the plugs carefully transferred into sterile bijou bottles by pushing them through from the top of the mould with a sterile pipette tip.

2.22.2 Lysis of DNA in agarose plugs

A 2 ml volume of lysis buffer (1% N-lauroylsarcosine, 50 mM Na_2EDTA, pH 9.5) containing 0.5 mg/ml of Proteinase K was added to each bijou and plugs were incubated at 56°C overnight in a shaking waterbath (Gallenkamp). After incubation, the lysis buffer was removed and replaced with fresh buffer containing the same concentration of Proteinase K, followed by another overnight incubation at 56°C. This procedure was repeated and after removal of the lysis buffer, the plugs were washed in 2 ml of TE buffer (10 mM Tris-HCl, 10 mM Na_2EDTA, pH 7.5) for 30 minutes at 4°C. The wash stage was repeated a further 2 times with fresh TE buffer used each time. The plugs were subsequently stored in TE buffer at 4°C until ready for use. (Plugs were stored under these conditions for several months with no DNA degradation if the TE buffer was replaced approximately every 6 weeks).

2.22.3 Digestion of DNA in agarose plugs

DNA plugs were transferred into a sterile petri dish where an appropriate portion (equivalent to the size of the well former being used) was cut using a sterile scalpel blade. Each plug piece was carefully transferred to a sterile bijou into which 4 ml of 1 x KGB buffer (2 x stock: 200 mM potassium glutamate, 50 mM tris-acetate pH 7.5, 20 mM magnesium acetate, 100 μg/ml bovine serum albumine fraction V, 1 mM β-mercaptoethanol in Milliq) had been added. The bijoux were incubated at 4°C for 2-3 hours with at least 2 changes of 1 x KGB buffer during this period.

The buffer was removed and the DNA plugs transferred to sterile Eppendorfs to which the following components were added in that order:

47 µl of MilliQ water
50 µl 2 x KGB buffer
1 µl 1mg/ml BSA (Promega UK)
2 µl 10U/ul *Apa* I restriction endonuclease (Promega UK)

The contents were mixed well by gently tapping the sides of the Eppendorf several times. The DNA was subsequently digested for 16 hours at 25°C, after which the restriction mixture was removed and the plug pieces carefully transferred to sterile 20 ml universal containers. MilliQ water (approximately 10 ml) was added to each universal to wash the DNA plugs. The universals were kept at 4°C for 1 hour, with at least 1 change of distilled water during this period. Plugs were stored at 4°C until the gel was ready to be loaded.

2.22.4 Preparation of agarose gel

The gel casting tray was wiped with absolute alcohol and assembled according to the manufacturer's instructions (Bio Rad). Tris-Borate-EDTA (TBE) buffer (Sigma) was diluted 1:10 to give a final concentration of x 0.5 in a 2 L volume. A 100 ml volume of this dilution was aliquoted into a sterile 250 ml Duran bottle (Schott), into which PFGE-grade agarose (Bio Rad) was added (final agarose concentration of 1%). The agarose solution was boiled at 100°C for 30 minutes and then allowed to cool to approximately 55°C. The cooled agarose (leaving approximately 2 ml in the bottle) was carefully poured into the assembled casting tray and allowed to set for at least 45 minutes, with the remaining 2 ml kept molten at 55°C. The rest of the diluted buffer was added to the electrophoresis cell (Bio Rad), and allowed to circulate with cooling until it reached the required temperature.

2.22.5 Loading of PFGE gel

Once the comb had been carefully removed from the gel, the plugs containing restricted DNA were carefully inserted into the appropriate wells using a sterile disposable plastic loop (Technical Service Consultants Ltd), ensuring that no air bubbles were introduced. DNA Size Standards (λ ladder range 48.5-970 kbp, Bio Rad) were also loaded into a well approximately half way along the gel. The wells (including those with no DNA plugs) were filled with the molten agarose and the gel was kept at 4°C until the agarose had set.

2.22.6 Running conditions

The gel was removed from the casting tray and placed in the electrophoresis cell. Gel running conditions were as follows:

Initial pulse time	1 sec	Run time	20 hours
Final pulse time	35 sec	Voltage	200 V (6V/cm)
Ratio	1.0	Temperatue	14°C

2.22.7 Gel staining and visualisation of RFLP patterns

The gel was carefully removed from the electrophoresis cell and transferred to a container to which 200 ml of ethidium bromide solution (0.5 mg/L) had been added. Staining was carried out with gentle agitation for approximately 30-45 minutes, followed by 2 x 15 minute washes in distilled water.

RFLP patterns were visualised on a UV transilluminator and photographed with a Polaroid camera fitted with a Tiffen orange filter.

2.22.8 Analysis of RFLP patterns

The relatedness of patterns was compared by the GelCompar v.3.10 computer program (Applied Maths, Kortrijk, Belgium). Photographs of RFLP patterns were scanned (ScanJet 4c, Hewlett Packard), and the files exported into the GelCompar program which generated a matrix of similarity coefficients between all pairs of strains using the Dice coefficient. Patterns were also analysed manually using the criteria of Tenover and Arbeit (1995).

CHAPTER 3

DETERMINATION OF RESISTANCE MECHANISMS IN CARBAPENEM-RESISTANT CLINICAL ISOLATES OF *ACINETOBACTER* FROM ARGENTINA

3.1 Strains

A total of 18 clinical isolates were collected from 3 hospitals in Buenos Aires, Argentina, all of which were reported to have reduced susceptibilities to imipenem (Table 3.1). One of the hospitals (CEMIC) isolated 5 imipenem-resistant strains within a 1 year period from October 1993 to November 1994. A year later, another 4 resistant strains were isolated, followed by an additional 4 in 1998. Three imipenem-resistant strains were also isolated from another teaching hospital in 1994, and another 2 from a geriatric hospital in the same year. Species identification by the API 20NE system indicated that the majority of isolates belonged to the *A. baumannii* complex.

3.2 Antimicrobial susceptibility testing

Table 3.2 lists the MIC values of a range of antibiotics tested against these isolates. A high percentage of isolates were resistant to all of the antibiotics tested, with 100% resistance demonstrated to amoxycillin, the β-lactam/inhibitor combination of amoxycillin and clavulanic acid, to ciprofloxacin, and to each of the cephalosporins tested.

The majority of isolates (89%) had MIC values of imipenem above the recommended breakpoint compared with 16% that had MIC values above the breakpoint for meropenem. However, nearly 78% of those that were 'susceptible' to meropenem had MIC values equal to the breakpoint. Gentamicin was the most effective antibiotic tested, with 50% of the isolates remaining susceptible. One isolate (number 789) had a similar resistance profile to the other strains; however, this strain was susceptible to both carbapenems (Table 3.2).

Table 3.1 Species identification and clinical details of *Acinetobacter* isolates from Buenos Aires, Argentina

Isolate	Identification	Date of Isolation	Hospital	Source
779	*A. baumannii*	02/10/93	CEMIC	Catheter site
780	*A. baumannii*	13/03/94	CEMIC	Catheter site
781	*A. baumannii*	02/05/94	CEMIC	BAL
782	*A. baumannii*	03/11/94	CEMIC	BAL
783	*A. baumannii*	21/07/95	CEMIC	BAL
784	*A. baumannii*	22/07/94	CEMIC	Catheter site
785	*A. baumannii*	1994	Hospital de Clinicas	BAL
786	*A. baumannii*	1994	Hospital de Clinicas	BAL
787	*A. junii*	1994	Hospital de Clinicas	Soft tissue
788	*A. baumannii*	21/08/94	Sanatorio Greyton	Urine
789	*A. baumannii*	1994	Sanatorio Greyton	BAL
790	*A. baumannii*	07/10/95	CEMIC	BAL
791	*A. baumannii*	16/10/95	CEMIC	BAL
883	*A. baumannii*	1995	CEMIC	BAL
884	*A baumannii*	07/98-08/98	CEMIC	BAL
885	*A. baumannii*	07/98-08/98	CEMIC	BAL
886	*A. baumannii*	07/98-08/98	CEMIC	BAL
887	*A. baumannii*	07/98-08/98	CEMIC	Catheter & blood culture

Abbreviations:

Teaching hospitals; CEMIC (Centro de Educación Médica e Investigaciones Clínicas), Hospital de Clinicas; Geriatric hospital, Sanatorio Greyton; BAL, bronchial alveolar lavage

Table 3.2 MIC values of *Acinetobacter* clinical isolates

	MIC value (mg/L)								
Isolate	**AMOX**	**CO-AMOX**	**CLD**	**CTX**	**CAZ**	**IMP**	**MER**	**CIP**	**GENT**
779	>128	128	>128	64	4	8	4	>16	0.5
780	>128	128	>128	64	4	8	4	>16	0.5
781	>128	>128	>128	64	4	8	4	>16	0.5
782	>128	128	>128	64	4	8	4	>16	8
783	>128	>128	>128	32	4	16	8	>16	1
784	>128	>128	>128	32	4	16	4	>16	2
785	>128	>128	>128	32	4	32	4	>16	2
786	>128	>128	>128	64	4	8	4	>16	>64
787	>128	>128	>128	32	4	16	4	>16	1
788	>128	128	>128	64	4	8	8	>16	>64
789	>128	32	>128	>128	>128	0.25	0.5	>16	>64
790	>128	>128	>128	64	128	16	4	>16	>64
791	>128	>128	>128	32	4	32	8	>16	>64
883	>128	>128	>128	64	64	4	4	>16	>128
884	>128	>128	>128	>128	>128	8	4	>16	0.12
885	>128	>128	>128	>128	>128	8	4	>16	0.12
886	>128	>128	>128	>128	>128	8	4	>16	0.12
887	>128	>128	>128	>128	>128	8	4	>16	0.25

Abbreviations: AMOX; Amoxycillin, CO-AMOX; Co-amoxiclavulanic acid, CLD; Cephaloridine, CTX; Cefotaxime, CAZ; Ceftazidime, IMP; Imipenem, MER; Meropenem, CIP; Ciprofloxacin, GENT; Gentamicin. **Recommended breakpoints** (mg/L) (Working Party on Antibiotic Sensitivity Testing, 1991 and 1998a): AMOX, CO-AMOX; 8, CLD, CAZ; 2, IMP, MER; 4, GENT, CTX, CIP; 1.

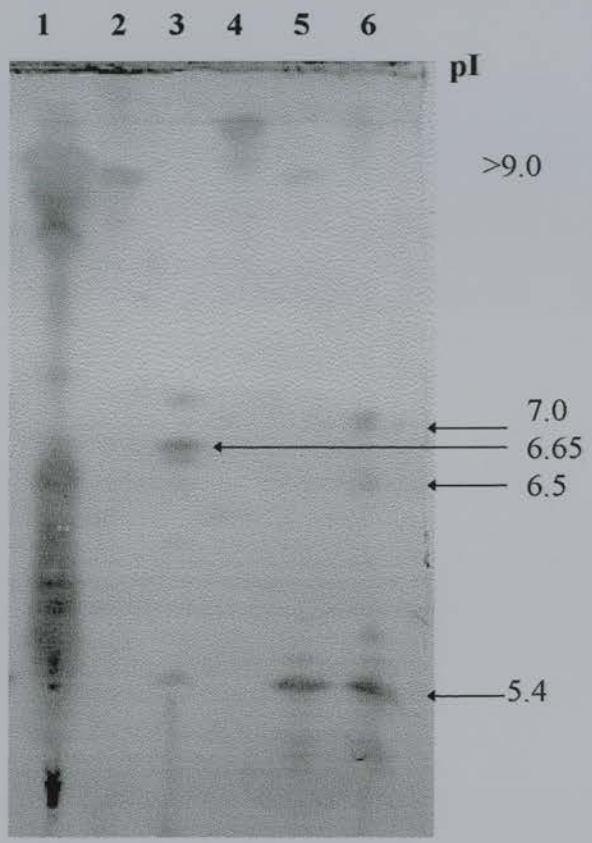
3.3 IEF analysis of carbapenem-resistant isolates

Crude small-scale β-lactamase extracts of strains with MIC values of 4 mg/L or above of imipenem were prepared and analysed by isoelectric focusing. The carbapenem-sensitive isolate (789) was also analysed by this method. The presence of 3 β-lactamases was revealed in the majority of imipenem-resistant strains (Figure 3.1 and Table 3.3). The first of these co-focused with a TEM-1 control at pI 5.4. The second focused at high pIs (>8.0) that were reminiscent of the chromosomal cephalosporinases described by Hood and Amyes (1991).

Two unknown β-lactamase bands focused together at pIs 6.5 and 7.0, the latter being the predominant band. These bands did not resemble any of the known β-lactamases that had been included as reference bands on the gel (Figure 3.1). Both the unknown bands and the high bands of pI >8.0 were present in all isolates. The TEM-1-like band of pI 5.4 was present in 11 of the 17 strains (Table 3.3). A TEM-1-like band was also detected in the imipenem-sensitive isolate 789, along with a band of pI >8.0 however, the bands of pIs 6.5 and 7.0 were not present in this isolate.

The β-lactamase extract of a representative strain (790) that contained all 3 β-lactamases was subsequently loaded onto another IEF gel and electrophoresed under the same conditions. The gel was then overlaid with inhibitor solutions to detect loss of β-lactamase activity. This method, although not quantitative, provided additional evidence for the presumptive identity of these enzymes.

Figure 3.1 IEF gel of representative strain 790 demonstrating β-lactamases present in clinical isolates of *Acinetobacter* from Argentina



Lane	Strain	β-lactamase(s)
1	*E. cloacae* NOR-1	NMC-A (pI 6.9)
2	*S. marcescens* S6	Sme-1 (pI 9.7)
3	*A. baumannii* 6B 92	ARI-1 (pI 6.65)
4	*P. aeruginosa* M18	Imp-1 (pI >9.5)
5	*E. coli* J62-2	TEM-1 (pI 5.4)
6	*A. baumannii* 790	pIs 5.4, 6.5, 7.0, >8.0

Table 3.3 Distribution of β-lactamase bands in clinical isolates of *Acinetobacter* spp.

	Carbapenem MICs (mg/L)		
Isolate	**Imipenem**	**Meropenem**	**β-lactamase bands (pI)**
779	8	4	7.0 (main band), >8.0
780	8	4	7.0, >8.0
781	8	4	7.0, >8.0
782	8	4	7.0 >8.0
783	16	8	5.4, 7.0, >8.0
784	16	4	5.4, 7.0, >8.0
785	32	4	5.4, 7.0, >8.0
786	8	4	5.4, 7.0, >8.0
787	16	4	5.4, 7.0, >8.0
788	8	8	7.0, >8.0
789	0.25	0.5	5.4, >8.0
790	16	4	5.4, 7.0, >8.0
791	32	8	5.4, 7.0, >8.0
883	4	4	7.0, >8.0
884	8	4	5.4, 7.0, >8.0
885	8	4	5.4, 7.0, >8.0
886	8	4	5.4, 7.0, >8.0
887	8	4	5.4, 7.0, >8.0

3.3.1 Inhibitor overlays

Table 3.4 lists the inhibitors and their specific inhibitory activities used in IEF overlays. Although not an inhibitor *per se*, imipenem was included primarily to detect

whether the β-lactamases were able to bind to this β-lactam which, if found bound to the active-site, would give an indication that it was a potential substrate.

Table 3.4 Characteristics of inhibitors and concentrations used as overlays of IEF gels

Inhibitor	Concentration	Inhibitory affect
BRL 42715	100 μM	Serine active-site β-lactamases
EDTA	1 mM	Metal ion chelator - inhibits metallo-β-lactamases
Clavulanic acid	1 mM	Class A but not class C β-lactamases (some class D β-lactamases)
Imipenem	100 μM	-

The activity of a standard TEM-1 β-lactamase (a class A serine active-site enzyme) that was run as a positive control was inhibited by clavulanic acid and BRL 42715, but not by EDTA, as expected (Figures 3.2 and 3.3). The β-lactamase extract of the L1 metallo-enzyme produced by *S. maltophilia* was also included and, as expected, its activity was inhibited by EDTA only. Partial inhibition of this enzyme was detected with imipenem. The TEM-1-like band of pI 5.4 in strain 790 was inhibited by clavulanic acid and BRL 42715, but not by EDTA, indicating that this β-lactamase was indeed TEM-derived. Imipenem had no effect on its activity, which suggested that the carbapenem is not a substrate of this enzyme.

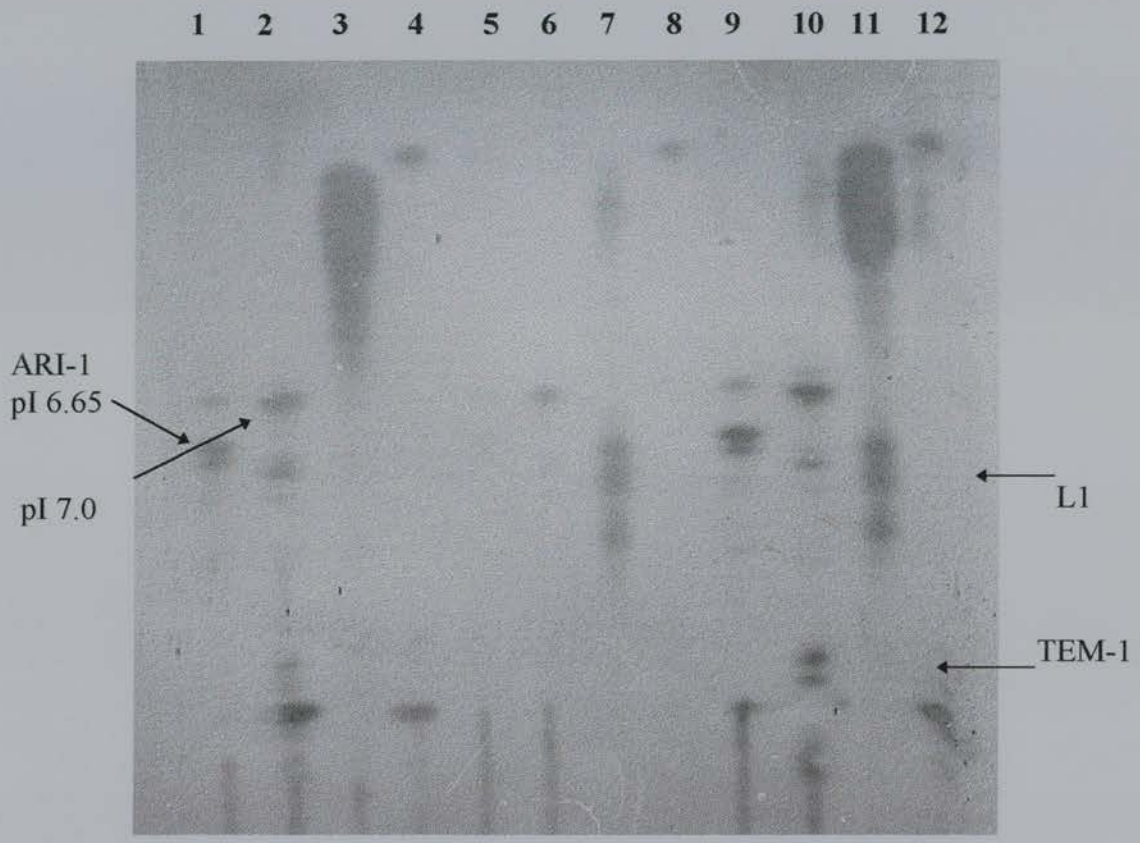
The bands of pI >8.0 focused rather weakly and therefore the inhibitory effects were difficult to read. However, they appeared to be inhibited by BRL 42715, and partially inhibited by imipenem, but not by EDTA. The bands of pI 6.5 and 7.0 were not inhibited by EDTA, indicating that this β-lactamase was not a metallo-enzyme. This was further substantiated by the inhibition of both bands by BRL 42715, an inhibitor of serine active-site β-lactamases. β-lactamase activity was also inhibited by imipenem, and partially inhibited by clavulanic acid. Although the latter result was

inconclusive, the possibility that this enzyme was a class A could not be ruled out at this stage. Inhibition by imipenem suggested that the enzyme was able to bind this β-lactam, and suggested its possible role as a mechanism of carbapenem resistance in those isolates that possessed this β-lactamase. Interestingly, the inhibitor profile of this β-lactamase was very similar to that of the serine active-site carbapenemase ARI-1 produced by strain 6B92 (Figures 3.2 and 3.3), which further supported this hypothesis.

3.4. Microbiological assays

β-lactamase extracts from all isolates that were resistant to imipenem were subjected to the microbiological assay using imipenem and meropenem antibiotic discs (both 10μg), to determine whether carbapenem inactivation occurred as a result of β-lactamase activity. The ARI-1 carbapenemase was included as a positive control, and the TEM-1 β-lactamase as a negative control. Loss of imipenem activity (indicated by a reduction in the zone diameter around the discs containing the β-lactamase dilutions), and meropenem activity (to a lesser extent) was demonstrated with all 17 imipenem-resistant isolates and with ARI-1, but not with TEM-1 or isolate 789, the carbapenem-sensitive isolate (Figure 3.4). This suggested that the β-lactamase(s) present in the imipenem-resistant isolates were able to hydrolyse and inactivate the carbapenems.

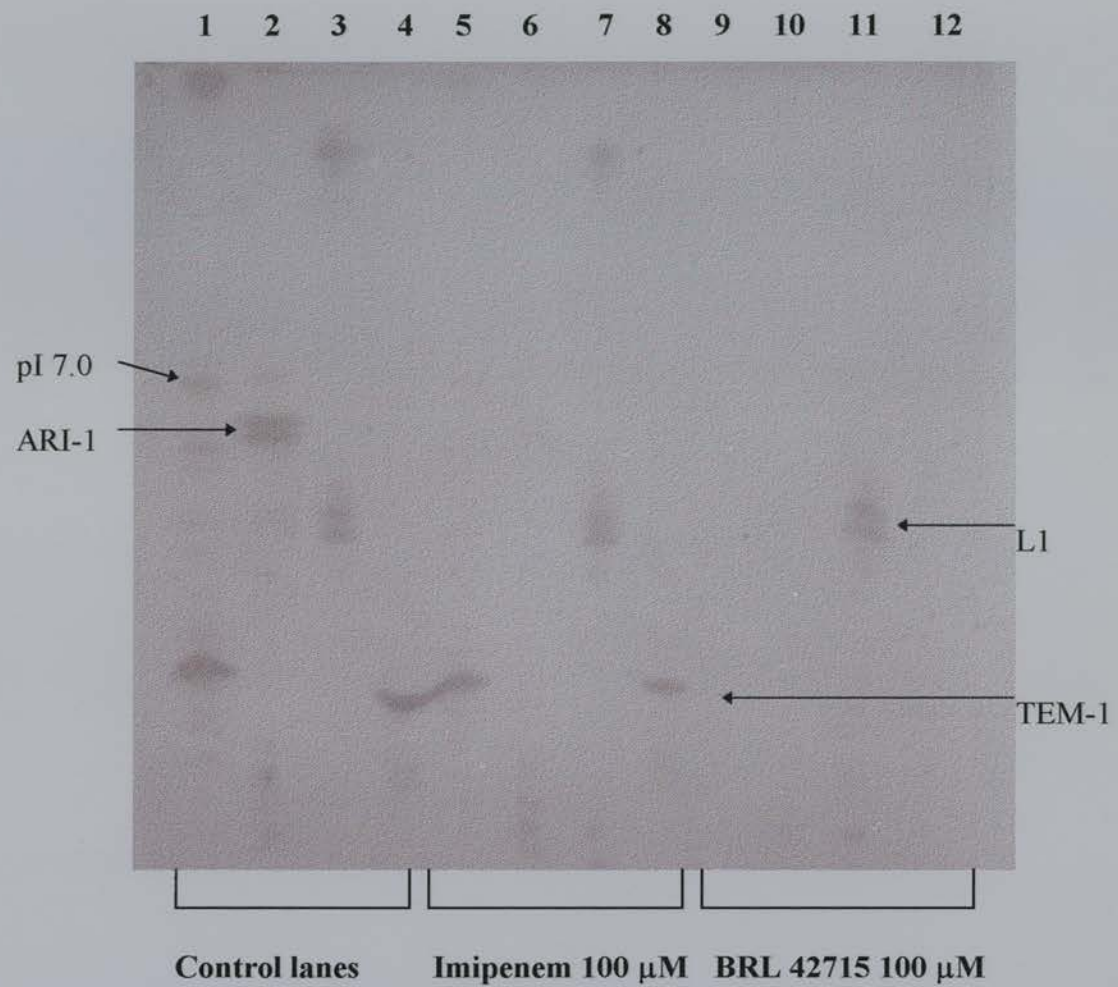
Figure 3.2 IEF demonstrating inhibitor overlays of β-lactamases in strain 790



Lane	Strain	β-lactamase
1	*A. baumannii* 6B92*	ARI-1
2	*A. baumannii* 790*	pI 5.4, 6.5, 7.0, >8.0
3	*S. maltophilia* ULA-511	L1
4	*E. coli* J62-2	TEM-1
5	As lane 1	
6	As lane 2	
7	As lane 3	
8	As lane 4	
9	As lane 1	
10	As lane 2	
11	As lane 3	
12	As lane 4	

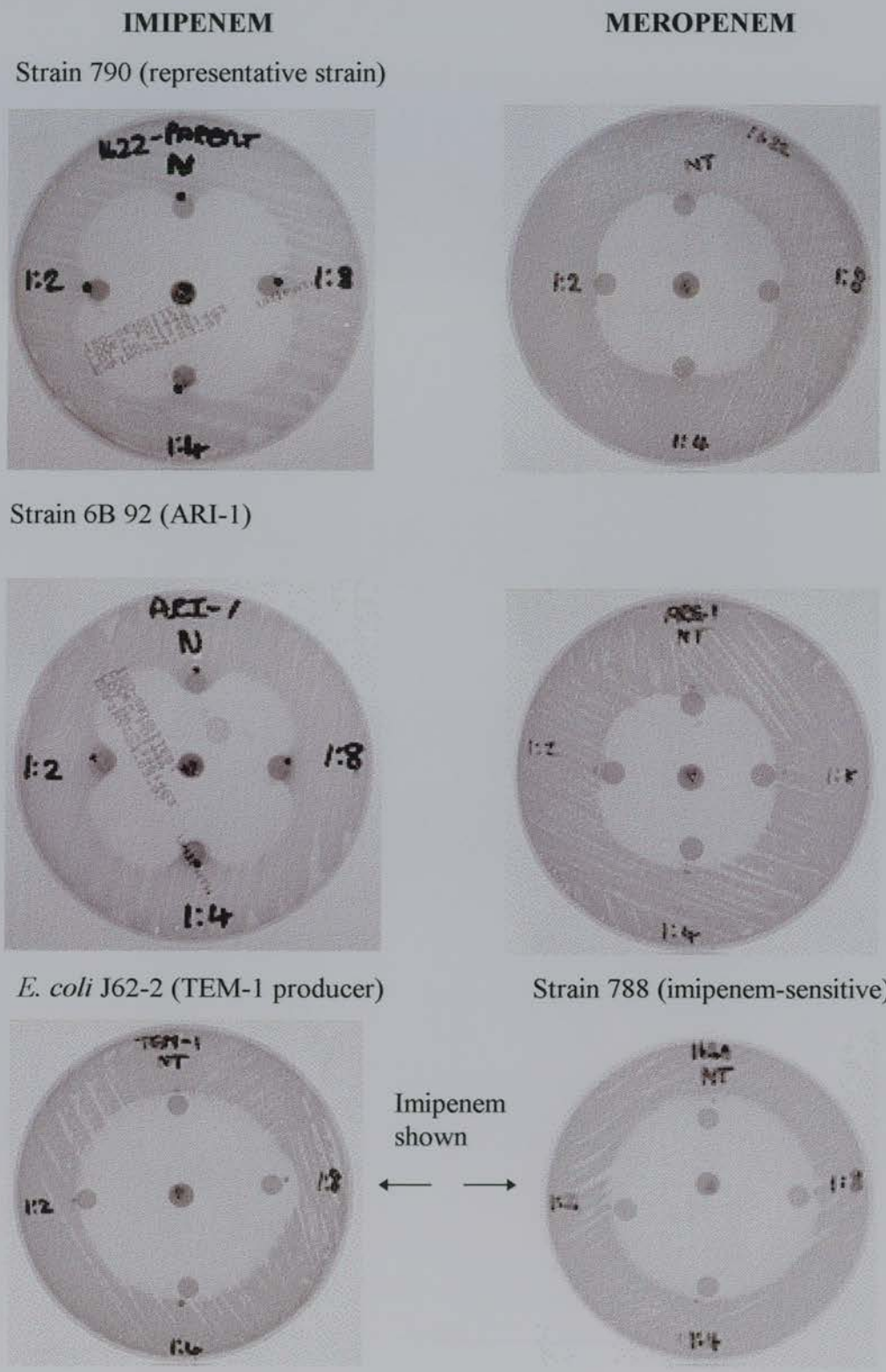
*pI >8.0 β-lactamases not visible in this photograph

Figure 3.3 IEF demonstrating inhibitor overlays of β-lactamases in strain 790



Lane	Strain	β-lactamase(s)
1	*A. baumannii* 790	pI 5.4, 6.5, 7.0, >8.0
2	*A. baumannii* 6B 92	ARI-1
3	*S. maltophilia* ULA-511	L1
4	*E. coli* J62-2	TEM-1
5	As lane 1	
6	As lane 2	
7	As lane 3	
8	As lane 4	
9	As lane 1	
10	As lane 2	
11	As lane 3	
12	As lane 4	

Figure 3.4 Microbiological assay demonstrating carbapenem inactivation by crude β-lactamase extracts



3.5 Induction studies of β-lactamases

Large-scale β-lactamase preparations of strain 790 and 6B 92 were prepared from broth cultures grown in the presence of imipenem at ¼ their respective MIC values. Aliquots of the broths were removed and the MIC values of imipenem determined. The crude preparations were subjected to spectrophotometric analysis to determine the hydrolysis rates of both nitrocephin and imipenem before and after induction. These results are summarised in Table 3.5. There was no increase in MIC value detected after the induction of either strain. Both induced strains had identical MIC values to the non-induced cultures (16 mg/L for 790 and 8 mg/L for 6B 92). In addition, spectrophotometric analysis did not demonstrate a significant difference in the hydrolysis rates of nitrocephin for induced and non-induced cultures (Table 3.5).

Table 3.5 MIC values and rates of hydrolysis of induced and non-induced cultures of strains 790 and 6B 92

Strain	**Imipenem MIC (mg/L)**	**Rate of hydrolysis (nmoles hydrolysed min $^{-1}$ mg $^{-1}$ protein)**		
		Nitrocephin (10^{-4} M)	**Pre-incubation imipenem (10^{-3} M)**	**Imipenem**
790	16	8.39	0.32	ND (<0.1)
790 induced	16	11.33	0.68	ND (<0.1)
6B 92	8	62.43	0.33	ND (<0.1)
6B 92 induced	8	67.76	0.68	ND (<0.1)
S. maltophilia ULA-511 (L1)	NT	NT	8.36	16.09

ND = not detected; NT = not tested

Direct hydrolysis of imipenem could not be detected by this method with both strains. The crude β-lactamase extract of an L1-producing strain was therefore assayed as a control to confirm that this was not as a result of inactivation of the imipenem.

The β-lactamase extracts were then incubated with imipenem for 10 minutes at 37°C before determining the hydrolysis rates for nitrocephin. There was a dramatic decrease in these rates with the β-lactamase extracts from both induced and non-induced cultures of 790 and 6B 92. With strain 790, hydrolysis rates decreased by 85% and 96% for induced and non-induced cultures respectively. Strain 6B 92 demonstrated a 99% reduction in nitrocephin hydrolysis with both induced and non-induced cultures. Pre-incubation with imipenem and the β-lactamase extract of the L1 producer resulted in a decrease of nitrocephin hydrolysis of 48%. Although direct imipenem hydrolysis could not be detected with the β-lactamase extracts of strains 790 and 6B 92, the pre-incubation assays with imipenem suggested that binding to imipenem had occurred, resulting in a loss of available enzyme and a subsequent decrease in the rate of nitrocephin hydrolysis.

3.6 Modified microbiological assay of crude extracts from strain 790 and strain 6B92

The crude β-lactamase extract of strain 790 did not appear to hydrolyse imipenem spectrophotometrically over a 5 minute period. Therefore the microbiological assay was adapted to determine whether slow hydrolysis occurred over a longer period of time. Crude β-lactamase extracts of strain 790 and 6B 92 were subjected to the assay as previously described. The rate of imipenem hydrolysis by strain 790 was 0.75 nmoles hydrolysed min^{-1} ml^{-1} of sample and the specific activity was calculated as 0.055 nmoles hydrolysed min^{-1} mg^{-1} of protein. Crude extract from strain 6B 92 which produces the ARI-1 carbapenemase, hydrolysed imipenem at a rate of 0.5 nmoles hydrolysed min^{-1} ml^{-1} of sample. The specific activity was calculated as

0.043 nmoles hydrolysed min $^{-1}$ mg $^{-1}$ of protein. Thus, crude β-lactamase extracts from both strains hydrolysed imipenem slowly at comparable rates.

3.7 Elimination of plasmid-determined resistance by treatment with ethidium bromide

The results of the microbiological assays suggested that carbapenem resistance in strain 790 and indeed in the other carbapenem-resistant isolates, may be associated with β-lactamase activity. Strain 790 was therefore subjected to treatment with ethidium bromide to investigate this hypothesis further. Strain 6B 92 which produces the plasmid-encoded ARI-1 β-lactamase, was included as a positive control. Following treatment, 14/38 (36.8%) of colonies of strain 790 failed to grow on IST agar plates containing a sub-inhibitory concentration of imipenem, which suggested that they had been cured of imipenem resistance.

A similar cure rate was obtained with colonies of 6B 92 (16/39, or 41%). Antimicrobial susceptibility tests of strain 790 cured colonies confirmed that the MIC value of imipenem had decreased to a susceptible level (Table 3.6). None of the other β-lactams tested had been affected by curing, as indicated by similar MIC values for both cured and parent colonies. These findings suggested that the genetic determinant responsible for carbapenem resistance was associated with a plasmid.

Table 3.6 MIC values of cured and parent colonies of strain 790 following treatment with ethidium bromide

	MIC values (mg/L)	
β-lactam	**790 parent**	**790 cured**
Imipenem	**16**	**0.5**
Meropenem	4	1.0
Amoxycillin	>128	>128
Amoxicillin/clavulanic acid	128	64
Cephaloridine	>128	>128
Cefuroxime	64	64
Cefotaxime	32	32
Ceftazidime	16	16

3.7.1 Analysis of the β-lactamase content of cured strain 790 by IEF

Crude β-lactamase extracts of several of the cured colonies were subjected to isoelectric focusing to ascertain whether loss of carbapenem resistance was associated with the loss of the unknown β-lactamase. All the assayed extracts had lost the β-lactamase bands at pI 6.5 and 7.0 (Figure 3.5), which provided strong evidence that this β-lactamase was indeed associated with plasmid-mediated carbapenemase activity. Both the TEM-1 β-lactamase and the cephalosporinase were still present in the extract from the cured colonies.

3.7.2 Microbiological assays of cured colonies of strain 790

To determine whether carbapenem inactivation was diminished after loss of the β-lactamase of pI 7.0, a crude β-lactamase extract of cured strain 790 was subjected to the microbiological and the modified microbiological assays. The extract failed to inactivate both carbapenems as determined by no reduction in the zone of inhibition

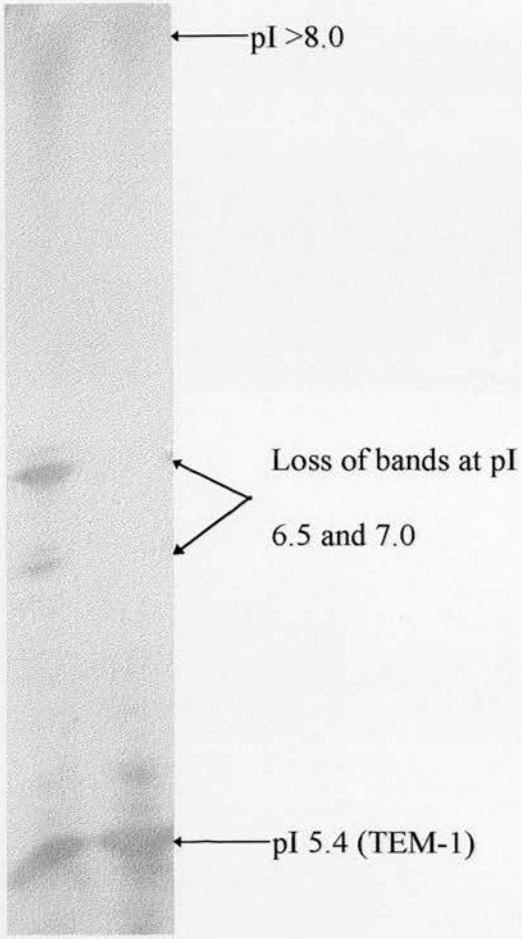
around the discs (Figure 3.5). In addition, there was an 11-fold decrease in the rate of imipenem hydrolysis with the β-lactamase extract of the cured strain compared with the parent (0.067 compared with 0.75 nmoles hydrolysed min^{-1} ml^{-1} of sample). There was also a corresponding reduction in the specific activity, calculated at 0.005 compared with 0.055 nmoles hydrolysed min^{-1} mg^{-1} of protein. These results provided further evidence that the β-lactamase of pI 7.0 lost after treatment with ethidium bromide was indeed associated with carbapenem resistance.

3.7.3 The effect of BRL 42715 on the MIC values of the carbapenems in strain 790

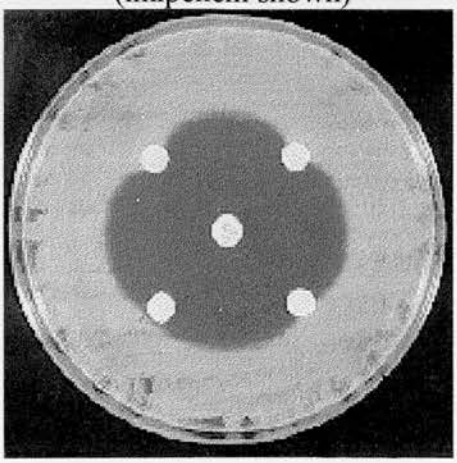
It now appeared that there was a novel carbapenemase present in the 17 isolates from Argentina. The curing results did not rule out the possibility however, that changes in outer membrane permeability or alterations in PBPs could still be contributing factors to carbapenem resistance. This was investigated by combining the serine active-site inhibitor BRL 42715 with both carbapenems in susceptibility tests (Table 3.7).

The results demonstrated a significant decrease in the MIC values of the imipenem/inhibitor combination obtained with the parent strain to a level comparable with the imipenem MIC value for the cured strain. There was not as significant a decrease in the MIC value of meropenem in the presence of the inhibitor. These results provided further support for the existing evidence that β-lactamase activity, and not changes in outer membrane permeability, was the major factor responsible for imipenem resistance. Furthermore, there was no significant change in MIC values observed with the cured strain, indicating that carbapenemase activity was not associated with the TEM or cephalosporinase present in this strain. The β-lactamase of pI 6.5 and 7.0 was subsequently designated ARI-2.

Figure 3.5 IEF gel and microbiological assay of crude β-lactamase extracts from parent and cured colonies of strain 790



Parent - carbapenem inactivation
(imipenem shown)



Cured - no inactivation
(imipenem shown)

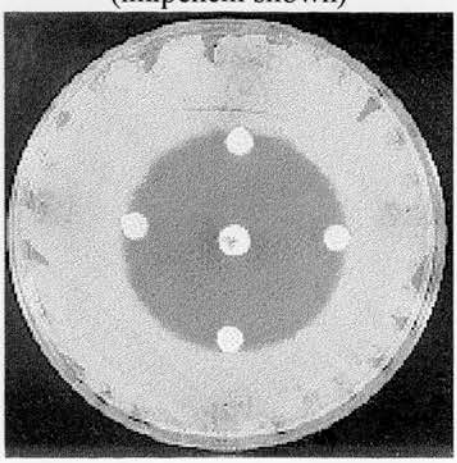


Table 3.7 MIC values of the carbapenems alone and in combination with BRL 42715

	MIC values (mg/L)			
Strain	**Imp**	**Imp/BRL**	**Mero**	**Mero/BRL**
790 parent	16	0.25	8	4
790 cured	0.5	0.25	1	1

Abbreviations: Imp; Imipenem, Imp/BRL; imipenem/BRL 42715 (4 mg/L fixed concentration), Mero; Meropenem, Mero/BRL; Meropenem/BRL 42715 (4 mg/L)

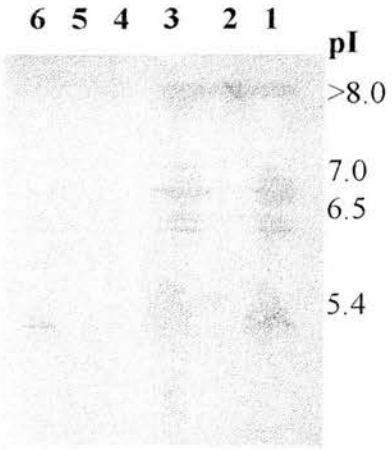
3.8 Partial purification of the ARI-2 β-lactamase

In order to ascertain the kinetic properties of ARI-2, anion-exchange and gel filtration chromatography were employed in an attempt to separate this enzyme from the other β-lactamases present in strain 790. A large-scale β-lactamase preparation of this strain was passed through a Mono Q anion-exchange column and fractions that displayed β-lactamase activity by the nitrocephin spot test were pooled and analysed by IEF (Figure 3.6). The ARI-2 and TEM-1 β-lactamases were detected in the first 13 ml volume eluted from the column before the NaCl gradient had been applied. These fractions also contained the cephalosporinase, although most of this β-lactamase remained bound to the column and was subsequently eluted in fractions 23-26 at approximately 125 mM NaCl.

Fractions 1-13 were applied to a Superdex 75 gel filtration column, and fractions were assayed for β-lactamase activity as previously described. Activity was demonstrated in pooled fractions 11-20 and in 29-40, which were both subjected to isoelectric focusing to determine their β-lactamase content. Both TEM-1 and ARI-2 were detected in fractions 29-40 (Figure 3.6). As the cephalosporinase was not present in these fractions, it was assumed that it would be present in fractions 11-20 however, this was

not apparent by IEF even though these fractions demonstrated β-lactamase activity by the nitrocephin spot test.

Figure 3.6 IEF of fractions collected from anion-exchange and gel filtration chromatography columns



Lane	Pooled fractions
1	1-13 from anion-exchange (unbound)
2	23-26 from anion-exchange (bound)
3	Strain 790 crude β-lactamase extract
4	Empty lane
5	11-20 from gel filtration
6	29-40 from gel filtration

3.8.1 Estimation of the molecular weight of ARI-2 by SDS-PAGE

Fractions 29-40 collected from the gel filtration column were subjected to SDS-PAGE to determine the molecular weight of the ARI-2 β-lactamase. As this procedure resulted in protein denaturation, the portion of the gel that contained the fractions was subjected to treatment with the non-ionic detergent Triton X-100 after electrophoresis, which returns protein molecules to their original conformation. The gel could then be

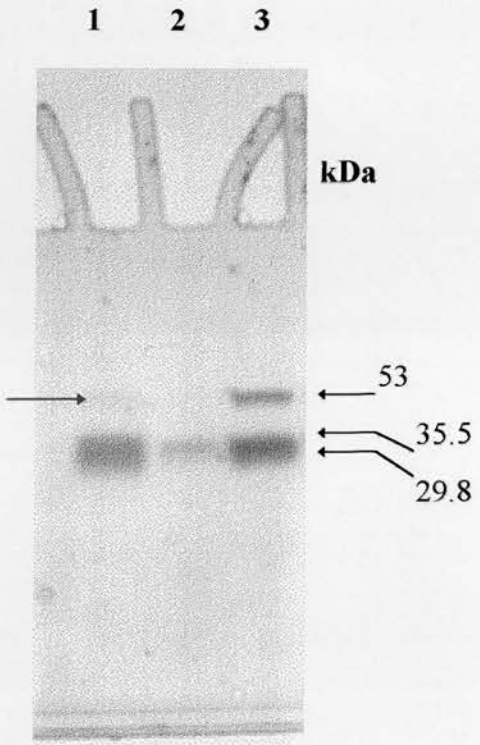
stained with nitrocephin to visualise the β-lactamases. Two diffused β-lactamase bands were visualised in close proximity to each other in fractions 29-40, which corresponded to those detected in the sample of crude extract that was included as reference (Figure 3.7). The molecular weights of these bands were determined by plotting a graph of the molecular weight of standard proteins (that had been electrophoresed on the other half of the gel and stained with Coomassie blue) against their migration distances through the gel. The molecular weights of the TEM-1 and ARI-2 β-lactamase bands were subsequently extrapolated from the graph. The molecular weight of the lower band was estimated as 29.8 kDa, and the band above it as 35.5 kDa. Surprisingly, a third, less prominent band which had not been detected by IEF, was also visible in these fractions, and which corresponded to the third β-lactamase band in the crude extract. The estimated molecular weight of this band was 53 kDa, and it was assumed to be the high molecular weight cephalosporinase. The band of 29.8 kDa was similar in weight to TEM-1, therefore the remaining band of 35.5 kDa was the ARI-2 β-lactamase.

As Figure 3.7 demonstrates, the TEM and ARI-2 β-lactamases proved to be close in molecular weight, which was probably the main reason why several attempts to separate them by chromatographic means had proved unsuccessful. In an attempt to circumnavigate this problem, the other 16 isolates were once again analysed to determine whether any of them expressed the ARI-2 β-lactamase to a comparable high level as strain 790, but also lacked the TEM β-lactamase. Strain 788 met these criteria and was subsequently used for further purification studies.

3.8.2 Partial purification of ARI-2 from strain 788

A 5 ml volume of a large-scale crude β-lactamase extract from strain 788 was passed through the Mono Q anion-exchange column as previously described. Fractions 2-9 contained predominantly the ARI-2 β-lactamase, but also some of the cephalosporinase. The majority of the latter enzyme, however, had bound to the column and was eluted in fractions 28-36 at approximately 120 mM NaCl. Fractions 2-9 were subsequently pooled and applied to the Superdex 75 gel filtration column.

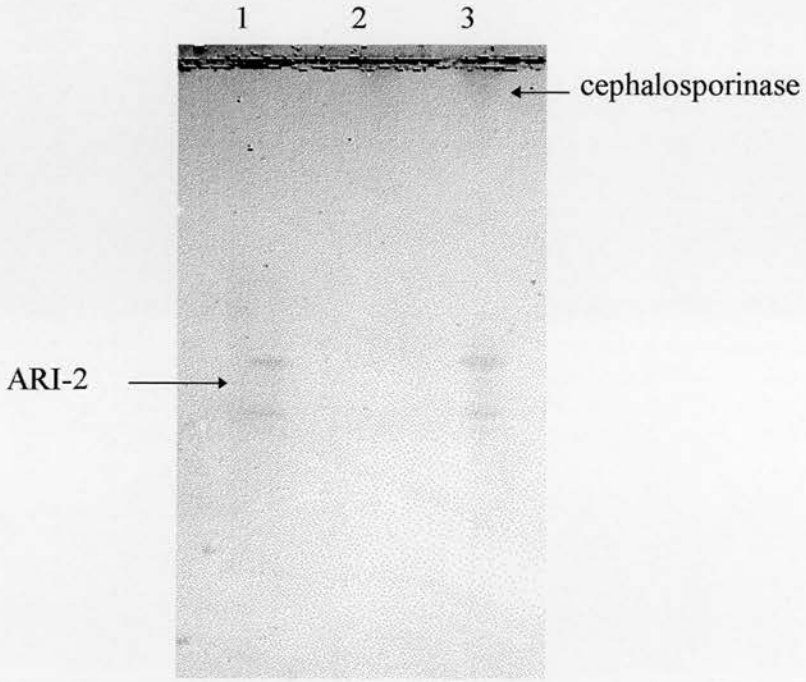
Figure 3.7 Nitrocephin activity stain of SDS-PAGE gel of fractions 29-40 collected from Superdex 75 gel filtration column



Lane	Sample
1	Fractions 29-34 Red arrow indicates position of 3rd band
2	Empty lane - *activity band due to carryover from lane 1
3	Crude β-lactamase extract from strain 790

IEF analysis of fractions collected from the column revealed that fractions 29-34 (which equated with eluate volumes of 123 ml and 131 ml) contained only the ARI-2 β-lactamase. Fractions 58-61 (eluate volumes of 167 ml and 172 ml) contained the cephalosporinase (Figure 3.8).

Figure 3.8 IEF gel of fractions collected from Superdex 75 gel filtration column



Lane 1; Fractions 29-34, **lane 2**; fractions 58-61, **lane 3**; crude β-lactamase extract of strain 788

3.9 Biochemical characterisation of ARI-2

The ARI-2 β-lactamase in fractions 29-34 was concentrated as previously described (section 2.11.3), and the rates of hydrolysis of a range of substrates, by the β-lactamase were determined by spectrophotometry. The specific activities were determined as previously described (section 2.12.2). V_{max} and K_m values were calculated from Lineweaver-Burk plots of initial velocity data. Ampicillin was the best substrate (specific activity of 36 nmoles hydrolysed min^{-1} mg^{-1} of protein compared with 1.9 nmoles of cephaloridine hydrolysed min^{-1} mg^{-1} of protein), which indicated that ARI-2 was predominantly a penicillinase. None of the cephalosporinases were hydrolysed, with the exception of cephaloridine. Interestingly, imipenem hydrolysis was detected with the purified enzyme extract. Although hydrolysis was slow, the enzyme had the

highest affinity for this substrate (k_m value of 11μM). No hydrolysis of meropenem was detected. Slow hydrolysis of both oxacillin and cloxacillin was also observed.

Table 3.8 Hydrolysis of β-lactam antibiotics by ARI-2

Substrate	Rate of hydrolysis[a]	Relative rate of hydrolysis[b]	Specific activity[c]	V_{max}[d]	K_m[e]	Relative V_{max}/K_m
Ampicillin	1923	100	36	1498.5	24	100
Cephaloridine	104	5.4	1.9	149.2	77	0.03
Cefuroxime	ND (<0.1)	-	-	-	-	-
Cefotaxime	ND (<0.1)	-	-	-	-	-
Ceftazidime	ND (<0.1)	-	-	-	-	-
Imipenem	125	6.5	4	46.8	11	0.068
Meropenem	ND (<0.1)	-	-	-	-	-
Oxacillin	61	3.2	1.3	484.1	531	0.015
Cloxacillin	64	3.3	1.2	31.9	129	0.004

[a] - nmoles of substrate hydrolysed min^{-1} ml^{-1} of enzyme solution
[b] - relative to ampicillin (100%)
[c] - nmoles of substrate hydrolysed min^{-1} mg^{-1} of protein
[d] - nmoles min^{-1} ml^{-1}
[e] - μM
ND = not detected

3.9.1 Inhibitor studies

Purified ARI-2 was pre-incubated for 10 minutes at 37°C with varying concentrations of inhibitor. Table 3.9 shows the concentration of each inhibitor required for 50% inhibition of enzyme activity (ID_{50}).

Table 3.9 ID_{50} values of inhibitors using nitrocephin (100μM) as reporter substrate

Inhibitor	ID_{50} (μM unless otherwise stated)
Clavulanic acid	100
EDTA	>1000
Cloxacillin	398
Sulbactam	50
Sodium chloride	3.2 mM
Imipenem	2.5
Meropenem	0.004

The metal ion chelator EDTA (10 mM) did not affect the activity of ARI-2, indicating that it was not a metallo-enzyme. Unfortunately, BRL 42715 was not available when these inhibitor studies were performed however, the IEF overlay of ARI-2 with this inhibitor (100 μM) completely inhibited its activity which, in addition to the lack of inhibition by EDTA, confirmed that it was a serine active-site β-lactamase (section 3.3.2). Sulbactam proved to be a more efficient inhibitor of enzyme activity than clavulanic acid. The class C inhibitor cloxacillin was not a good inhibitor of enzyme activity.

Enzyme activity was inhibited by chloride ions (ID_{50} value of 3.2 mM sodium chloride). Both imipenem and meropenem achieved 50% inhibition at 2.5 μM and 0.004 μM respectively, which indicated high affinity of ARI-2 for the carbapenems.

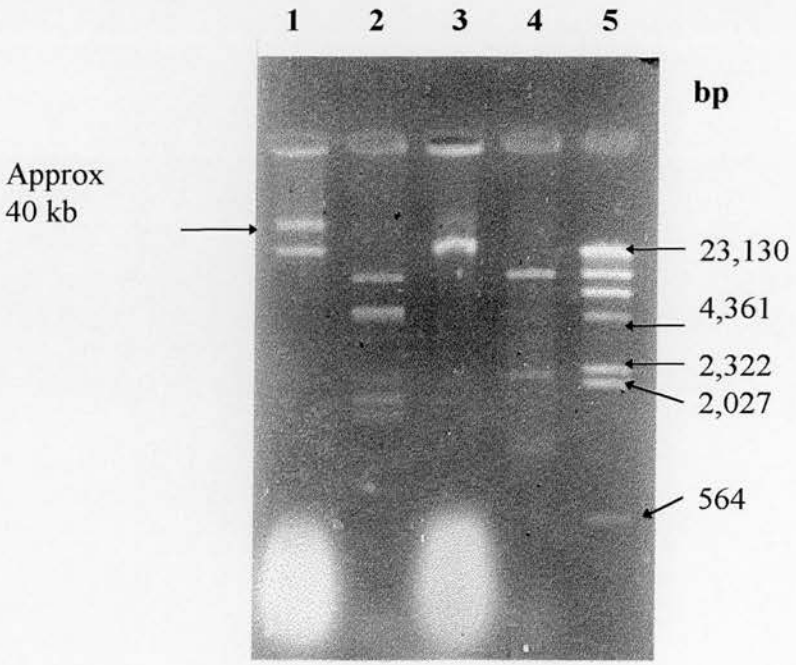
3.10 Attempts to visualise the plasmid carrying the *bla*$_{ARI-2}$ gene

Plasmid DNA from both parent and cured colonies of strain 790 were subjected to restriction endonuclease digestion with *Hin*d III enzyme as described in section 2.18. Following restriction, visualisation of fragments along with unrestricted plasmid DNA

demonstrated the presence of a DNA band in the parent strain that was not visible in the cured strain (Figure 3.9). This suggested that elimination of this plasmid was associated with a concurrent reduction in carbapenem MIC values to susceptible levels, and loss of the ARI-2 β-lactamase.

From the migration distances of the fragments, the plasmid visualised in strain 790 was estimated to be approximately 40 kb, although this was probably an underestimate since many of the fragments were difficult to visualise. However, when the plasmid was viewed alongside the bla_{ARI-1}-containing pUK1356 plasmid of the *Acinetobacter* sp. transconjugant (Figure 3.12), this estimate compared favourably with the latter, which is approximately 58 kb in size.

Figure 3.9 Visualisation of plasmid DNA obtained from parent and cured colonies of strain 790



Lane 1; plasmid DNA from strain 790, **lane 2**; 790 restricted with *Hin*d III, **lane 3**; plasmid DNA from cured strain 790, **lane 4**; cured 790 restricted with *Hin*d III, **lane 5**; λ/*Hin*d III fragments

3.11 Analysis of the mechanism of plasmid transfer

3.11.1 Conjugation studies

A series of conjugation experiments were performed using the rifampicin-resistant recipient *Acinetobacter* sp. BD413-2 in an attempt to determine whether the plasmid observed in strain 790 was conjugative. For both broth and membrane filter mating experiments at both mating temperatures, no transconjugant colonies were visible on nutrient agar plates containing imipenem at 4 mg/L and rifampicin at 50 mg/L.

3.11.2 Transformation of *Acinetobacter* sp. BD413-2 with plasmid DNA of *A. baumannii* strains 788 and 790

Following the failure to demonstrate conjugative transfer of the plasmid, plasmid DNA from strains 788 and 790 were used in an attempt to transform a naturally competent plasmid-free strain of *Acinetobacter* (BD413-2) according to the method described in section 2.19. For both plasmid DNA preparations, no colonies were visible on the selective IST agar containing 32 mg/L of rifampicin and 1 ml/L of imipenem even after a prolonged incubation period of 72 hours.

3.12 Investigation of the characteristics of bla_{ARI-2}

A series of PCR reactions were employed to investigate whether there were any homologous DNA regions shared between the gene encoding the ARI-2 gene and other β-lactamases.

3.12.1 PCR analysis with SHV-specific primers

Interestingly, it had been observed by IEF that the ARI-2 β-lactamase focused at an identical pI value to that of the SHV-3 β-lactamase (main band of pI 7.0). The biochemical analysis of ARI-2 had not indicated that it was SHV-derived; however,

PCR amplification with SHV-specific primers, and DNA hybridisation with an SHV gene probe were performed to rule out this possibility. Both experiments revealed no sequence homology, which demonstrated that ARI-2 was not an SHV β-lactamase. Attention was subsequently directed towards other β-lactamases.

3.12.2 PCR analysis with OXA-specific primers

The biochemical profile of ARI-2 had revealed that it hydrolysed both oxacillin and cloxacillin albeit slowly therefore, the possibility existed that it could potentially be related to the molecular class D β-lactamases. PCR amplification was subsequently performed with intragenic primers that had been designed from the $bla_{\text{OXA-21}}$ sequence of the oxacillin-hydrolysing β-lactamase OXA-21, which shares an identical pI to that of ARI-2. As the former enzyme had also been found in a clinical isolate of *A. baumannii,* the possibility existed that the ARI-2 β-lactamase could be a mutated form of this enzyme that had acquired the ability to hydrolyse imipenem.

Template DNA from strain 788, and from both parent and cured colonies of strain 790 was included in the reaction. Both OXA-21 and OXA-2 possess the non-silent mutation Ile-217 to Met (Vila *et al.*, 1997a) therefore, an OXA-2-producing strain (*E. coli* K12) was used as a positive control. The imipenem-sensitive strain 789 and a TEM-1-producing strain (*E. coli* J62-2) were included as negative controls. Gel electrophoresis of PCR products revealed the expected band of 550 bp with the OXA-2-producing strain (Figure 3.10). No bands were visualised with strains 788, 790 or with the negative control strains.

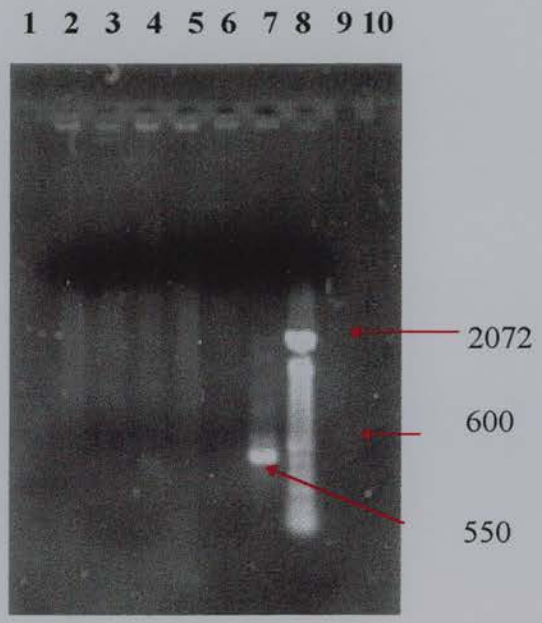
3.12.3 PCR analysis with ARI-1-specific primers

To investigate the degree of homology (if any) between the ARI-1 and ARI-2 β-lactamase genes, template DNA from strains 788 and 790 was subjected to PCR analysis with ARI-1-specific primers. The primers (Helen 3 and Helen 4) amplified a fragment of 513 bp within the $bla_{\text{ARI-1}}$ gene. Strains 6B 92 and *Acinetobacter* sp.

BD413-2 transconjugant were included as positive control strains. A TEM-1-producing strain (*E. coli* J62-2) and the imipenem-sensitive clinical strain 789 were also included as negative controls.

A PCR product of the anticipated size was obtained with both the clinical and transconjugant strains containing ARI-1 as expected. No products were visualised with strains 788 and 790, or with either of the negative controls (Figure 3.11).

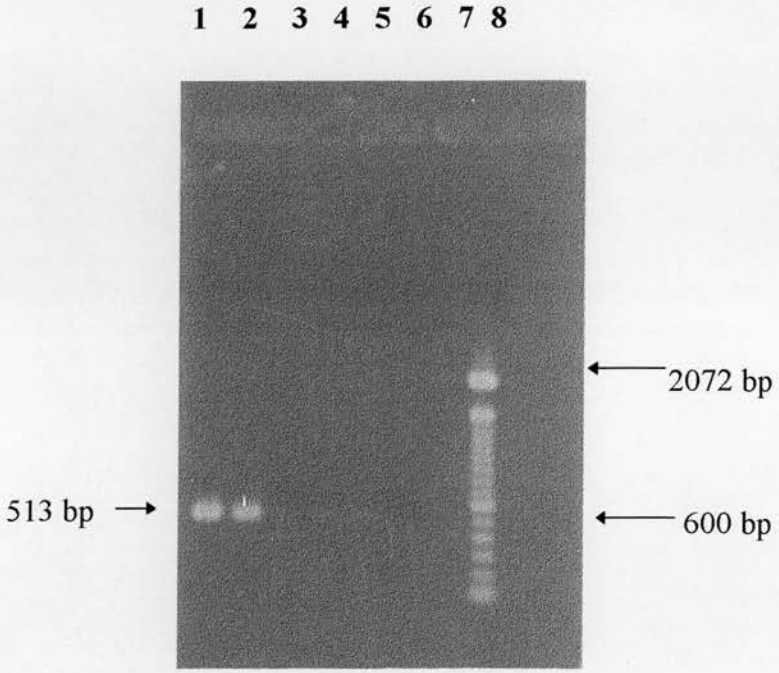
Figure 3.10 PCR analysis of *bla*$_{ARI-2}$-positive strains with *bla*$_{OXA-21}$- specific primers



Lane

1	*A. baumannii* strain 788
2	*A. baumannii* strain 790
3	Cured strain 790
4	Empty lane
5	Strain 789 (imipenem-sensitive) - negative control
6	*E. coli* J62-2 (TEM-1 producer) - negative control
7	*E. coli* K12 (OXA-2 producer) - positive control
8	100 bp λ ladder

Figure 3.11 PCR analysis of *bla*$_{ARI-2}$-positive strains with intragenic *bla*$_{ARI-1}$ primers



Lane	
1	*A. baumannii* 6B 92
2	*Acinetobacter* sp. BD413-2 transconjugant
3	*A. baumannii* strain 788
4	*A. baumannii* strain 790
5	*A. baumannii* strain 789 (imipenem-sensitive)
6	*E. coli* J62-2 (TEM-1 producer)
7	Primer control (no DNA template)
8	100 bp λ ladder

3.12.4 PCR amplification with ARI-1-specific primers utilising reduced annealing temperatures

A series of PCR reactions was performed with lower annealing temperatures in order to reduce primer specificity and encourage their annealing to DNA sequences of lower homology. If this proved successful, and such DNA sequences were amplified, the possibility existed that one of the products may have been generated from the template

DNA of strain 790. If this was the case, there was a chance (although somewhat slim) that the product would contain the ARI-2 gene.

An annealing temperature range of 50°C-33°C was employed. However, no products were visualised other than the anticipated product of 513 bp from the ARI-1-containing strains.

3.12.5 Hybridisation with intragenic gene probe for bla_{ARI-1}

PCR amplification of bla_{ARI-1} was performed with template DNA from the *Acinetobacter* sp. BD413-2 transconjugant strain using primers Helen 3 and 4 to amplify the 513 bp fragment within the gene. The PCR product was analysed by gel electrophoresis (Figure 3.12) to confirm that it was the anticipated size, and purified by the method described in section 2.20.5. Plasmid DNA obtained from the parent and cured colonies of strain 790 was restricted with *Hin*d III endonuclease (Figure 3.11) and covalently linked to a piece of nylon membrane (section 2.21.2).

Plasmid DNA from the *Acinetobacter* sp. BD413-2 transconjugant was also included as a positive control. A negative control in the form of genomic DNA from a TEM-1-producing *E. coli* strain (J62-2) was added to the membrane as a dot blot (section 2.21.1). This was also performed with chromosomal DNA from strain 790. Following hybridisation with the labelled probe, the membrane was subjected to high, medium and low stringency washes (section 2.21.5).

After the high stringency wash, the intragenic gene probe hybridised strongly with restricted DNA fragments and non-restricted plasmid DNA from the transconjugant of bla_{ARI-1} as expected (Figure 3.13). No other signals were detected. However, the plasmid DNA from strain 790 (that corresponded to the plasmid identified as potentially carrying bla_{ARI-2}) gave a positive signal after medium (indicated by red arrow in figure 3.13) and low stringency washes (Figure 3.13) indicating that there was some degree of homology with the ARI-1 β-lactamase gene. The probe also hybridised

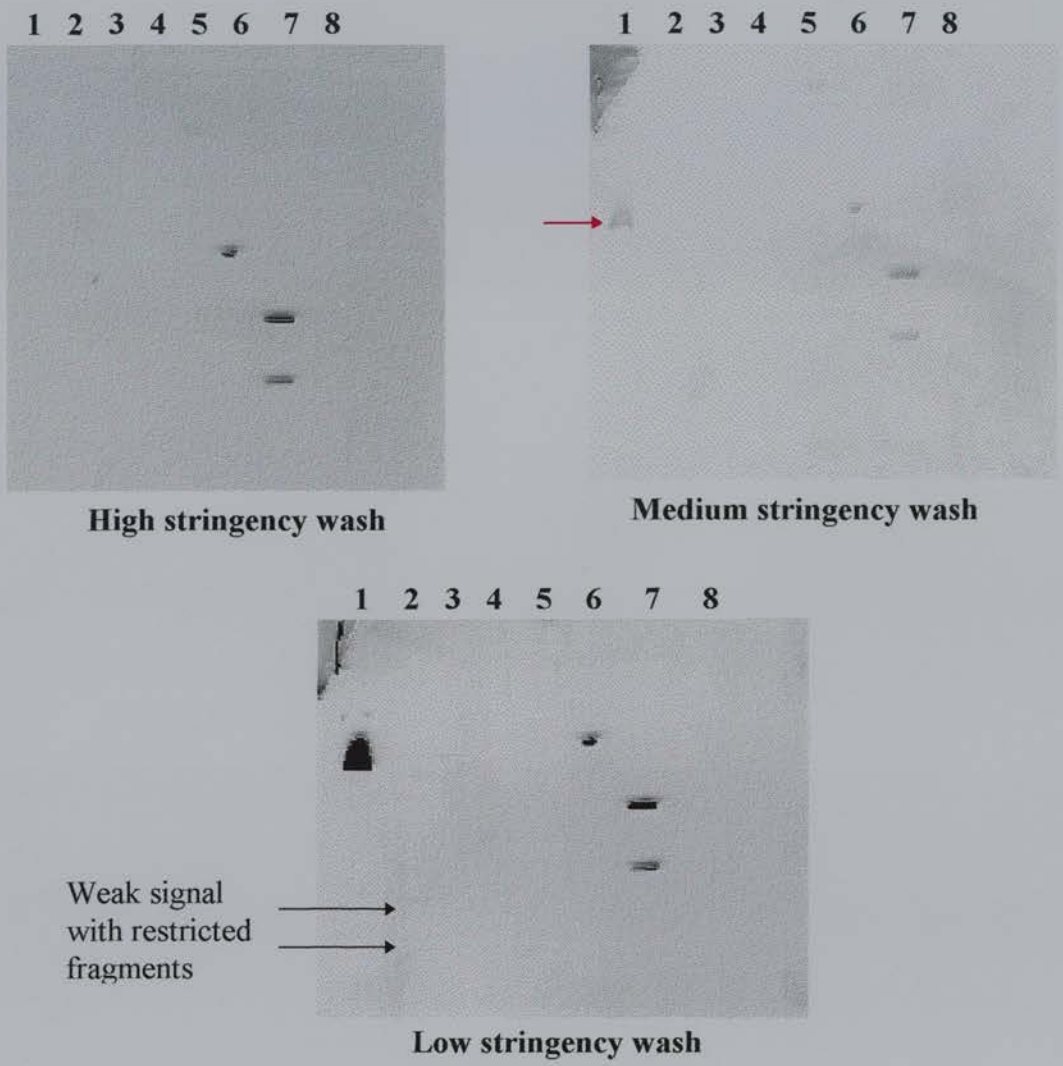
albeit very weakly, with 2 restricted plasmid DNA fragments from this strain (as indicated by the arrows in Figure 3.13). No signals were detected with plasmid DNA from the cured colony of strain 790, or with chromosomal DNA from this strain.

Figure 3.12 Electrophoresis of DNA used in hybridisation with *bla*$_{ARI-1}$ intragenic probe

Lane

1	*A. baumannii* strain 790 - plasmid DNA
2	*A. baumannii* strain 790 - plasmid DNA restricted with *Hin*d III
3	*A. baumannii* strain 790 cured - plasmid DNA
4	*A. baumannii* strain 790 cured - plasmid DNA restricted with *Hin*d III
5	λ DNA/*Hin*d III fragments
6	*Acinetobacter* sp. BD413-2 transconjugant plasmid DNA
7	*Acinetobacter* sp. BD413-2 transconjugant plasmid DNA restricted with *Hin*d III
8	λ DNA/*Hin*d III fragments

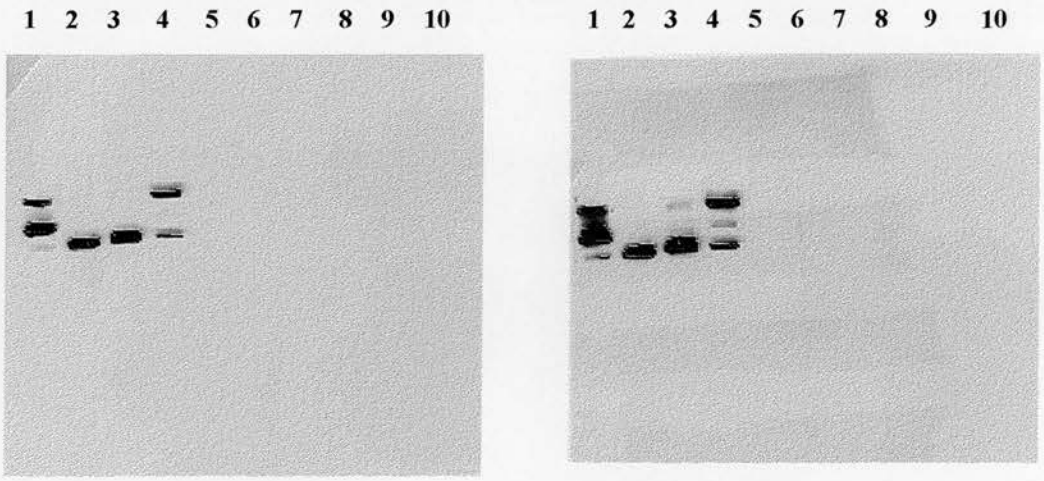
Figure 3.13 Hybridisation of *bla*$_{ARI-2}$-positive strain 790 with intragenic gene probe for *bla*$_{ARI-1}$



Lane	
1	Plasmid DNA - strain 790
2	*Hin*d III restricted plasmid DNA - strain 790
3	Plasmid DNA - cured strain 790
4	*Hin*d III restricted plasmid DNA - cured 790
5	λ/*Hin*d III fragments
6	*Acinetobacter* sp. BD413-2 transconjugant plasmid DNA
7	*Hin*d III restricted plasmid DNA - BD413-2
8	λ/*Hin*d III fragments

To determine whether a stronger hybridisation signal could be produced after restriction with endonucleases other than *Hin*d III, plasmid DNA from strain 790 was restricted with *Eco*R I, *Pst* I and *Bam*H I and hybridised with the intragenic bla_{ARI-1} probe. Restricted plasmid DNA from the transconjugant *Acinetobacter* sp. BD413-2 was again included as a positive control. The membrane was subjected to medium and high stringency washes. On this occasion however, both the restricted fragments of plasmid DNA and non-restricted plasmid DNA obtained from strain 790 failed to hybridise with the intragenic probe, even at low stringency (Figure 3.14). Strong signals were detected with plasmid DNA from the transconjugant after both stringency washes, indicating that the hybridisation procedure had been successful.

3.14 Hybridisation of bla_{ARI-2}-positive strain 790 with intragenic gene probe for bla_{ARI-1} following restriction with *Eco*R I, *Pst* I and *Bam*H I



Low stringency wash **Medium stringency wash**

Lane		Lane	
1	BD413-2 transconjugant plasmid DNA	6	790 Plasmid DNA
2	*Eco*R I restricted BD413-2	7	*Eco*R I restricted 790
3	*Pst* I restricted BD413-2	8	*Pst* I restricted 790
4	*Bam*H I restricted BD413-2	9	*Bam*H I restricted 790
5	λ/*Hin*d III fragments	10	λ/*Hin*d III fragments

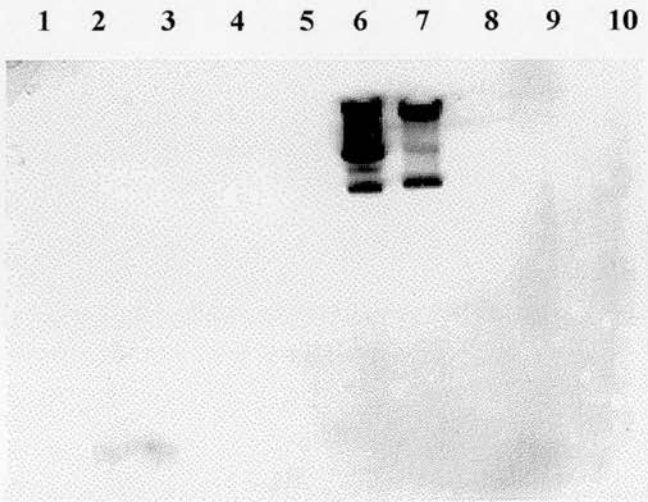
To confirm this result, the membrane was stripped and reprobed using the same hybridisation conditions as before. Again, the plasmid DNA from strain 790 failed to produce a signal. The membrane that had initially demonstrated hybridisation of the intragenic probe with this DNA preparation (Figure 3.13) was subsequently subjected to the same hybridisation procedure again to validate the original result. A positive signal was once again detected with the plasmid DNA from this strain and with the positive control, indicating that the initial result had been correct.

Different plasmid DNA preparations from strain 790 had been used for each of the hybridisation experiments therefore, a possible explanation for these opposing results was that the plasmid DNA used in the first hybridisation could have been contaminated with chromosomal DNA, and it was this DNA that the probe was hybridising to. Arguably, a dot blot of chromosomal DNA from the same strain had previously failed to hybridise with this probe (not shown in Figure 3.13) however, this may have been as a result of inadequate binding of DNA to the membrane.

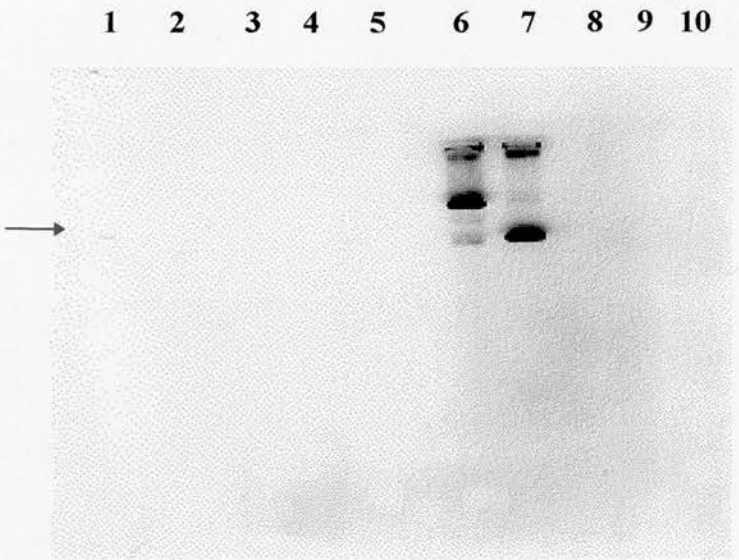
To investigate this hypothesis further, chromosomal and plasmid DNA samples were extracted from strain 790, restricted and transferred to a piece of membrane. Hybridisation was carried out with the intragenic probe as before, followed by washes at low and medium stringencies. Strong signals were detected with plasmid DNA from the *Acinetobacter* sp. transconjugant (both restricted and non-restricted) at both stringencies as expected (Figure 3.15). No signal was detected with plasmid DNA from strain 790. However, the probe hybridised very weakly with chromosomal DNA from this strain at low stringency (indicated by the arrow in Figure 3.15). This appeared to suggest that chromosomal contamination could indeed have produced the initial result. However, on this occasion, the signal was very much weaker despite the fact that sufficient chromosomal DNA had been transferred to the membrane.

Figure 3.15 Hybridisation of chromosomal and plasmid DNA from *bla*$_{ARI-2}$-positive strain 790 with intragenic gene probe for *bla*$_{ARI-1}$

A



B



A		**B**	
Lane		**Lane**	
1	Strain 790 plasmid DNA	1	Strain 790 chromosomal DNA
2	Strain 790 plasmid/*Eco*R I	2	Strain 790 chromosomal/*Eco*R I
3	Strain 790 cured plasmid DNA	3	Strain 790 cured chromosomal DNA
4	Strain 790 cured plasmid/*Eco*R I	4	Strain 790 cured chromosomal/*Eco*R I
5	GeneRuler DNA fragments	5	GeneRuler DNA fragments
6	ARI-1 transconjugant plasmid DNA	6	ARI-1 transconjugant plasmid DNA
7	ARI-1 transconjugant plasmid/*Eco*R I	7	ARI-1 transconjugant plasmid DNA/*Eco*R I
8	*E. coli* K12 (OXA-2) plasmid DNA	8	*E. coli* K12 (OXA-2) plasmid DNA
9	*E. coli* K12 (OXA-2) plasmid/*Eco*R I	9	*E. coli* K12 (OXA-2) plasmid/*Eco*R I
10	GeneRuler DNA fragments	10	GeneRuler DNA fragments

Faint signal with chromosomal DNA from strain 790 indicated by red arrow

If the ARI-2 β-lactamase gene was now located on the chromosome, as these results suggested, this was in contrast to the curing experiments performed with strain 790, which had demonstrated that plasmid elimination had resulted in the concurrent loss of ARI-2 from the cured strain. This evidence was further supported by visualisation of a large plasmid in the parent strain that was not present in the cured.

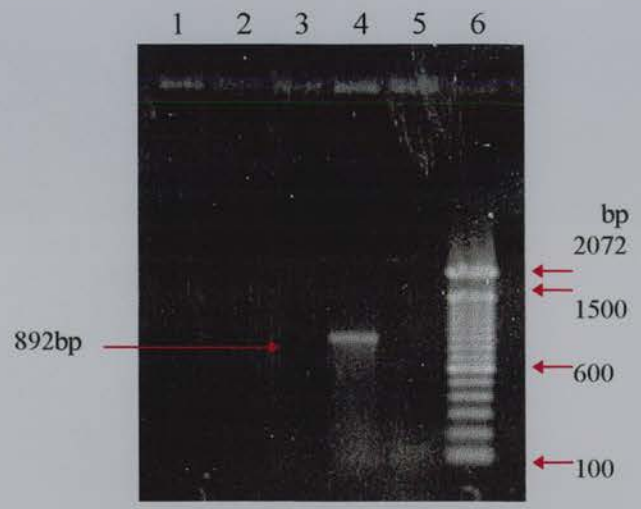
3.12.6 PCR analysis with class 1 integron-specific primers

A possibility existed that bla_{ARI-2} may be associated with a transposable element that facilitated its transfer between the chromosome and plasmid. To investigate this hypothesis, PCR amplification was employed with primers (intA and intB), which were designed to anneal to the 5' and 3' ends of the *intI 1* gene (i.e. the integrase gene located in the 5' conserved segment of class 1 integrons). Amplification with primers cass 1 and cass 2 was also performed. This primer pair was designed to amplify the variable region of class 1 integrons into which cassettes are integrated.

A positive control was employed in the form of template DNA from *E. coli* J53. This strain possessed a plasmid (pR388) which contained the *dhfr*IIb trimethoprim resistance gene located on one of 2 cassettes within a class 1 integron. Another *E. coli* J53 strain containing the plasmid pR483 which has no associated integron structure, was employed as a negative control.

As expected, PCR amplification with the int primers amplified a PCR product of 892 bp with pR388, indicating the presence of the *intI 1* gene (Figure 3.16). However, no product was detected with this plasmid using the cass primers when a product of approximately 1.5 kb was expected. A possible explanation was that the PCR conditions used were not suitable for amplifying products of that size. Both PCR reactions failed to amplify DNA from the ARI-2-producing strains 790 (both parent and cured) and 788. This suggested that there were no class 1 integrons present in these strains.

Figure 3.16 PCR amplification of *bla*$_{ARI-2}$-positive strains with class 1 integron-specific primers



Lane

1	Strain 790
2	Strain 790 cured colonies
3	Strain 788
4	*E. coli* J53 (pR388)
5	*E. coli* J53 (pR483)
6	GeneRuler DNA ladder

3.12.7 Hybridisation with degenerate probe for conserved sequence of *bla*$_{OXA}$ genes

Chromosomal and plasmid DNA from strain 790 was subjected to hybridisation with a degenerate oligonucleotide probe (ARI-NB) that had been designed from alignments of several class D β-lactamases (Figure 3.17).

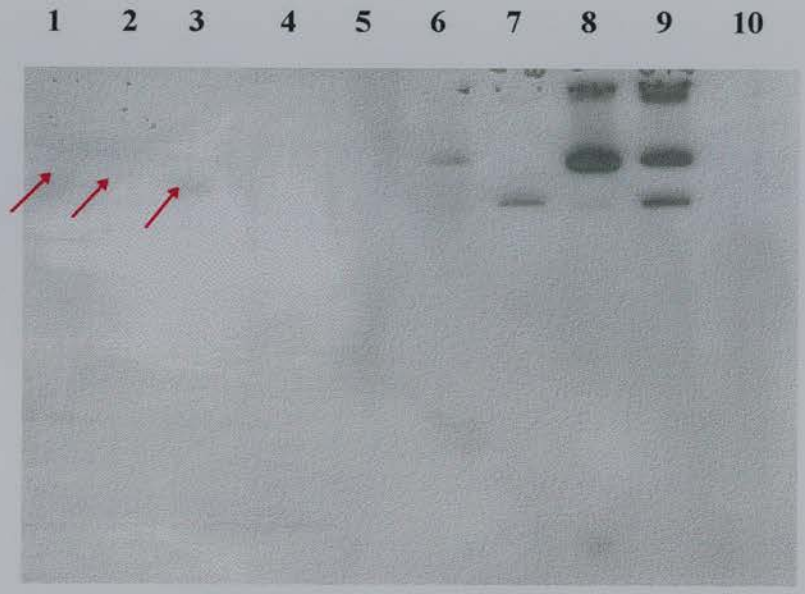
Figure 3.17 Amino acid sequence alignments of conserved sequence in molecular class D β-lactamases

Oxa-7	ES--NPGV**AWWVGWVE**KGAE-VYFFAFNMD	240
Oxa-13	ES--NPGV**AWWVGWVE**KGTE-VYFFAFNMD	240
Oxa-10	ES--NPGV**AWWVGWVE**KETE-VYFFAFNMD	240
Oxa-11	ES--NPGV**AWWVGWVE**KETE-VYFFAFNMD	240
Oxa-5	ES--SPGV**AWWVGWVE**KGTE-VYFFAFNMD	240
Oxa-2	-------M**GWWVGWVE**WPTG-SVFFALNID	238
Oxa-15	-------M**GWWVGWVE**WPTG-SVFFALNID	238
Oxa-21	-------M**GWWVGWVE**WPTG-PVFFALNID	238
ARI-1	-I--KPQV**GWLTGWVE**QPDGKIVAFALNME	249
Oxa-1	-R--TLQN**GWFEGFII**SKSG-HKYVFVSAL	249
Oxa-18	AKGGKAPI**GWGIGWAT**RDDRRVVFARLTVG	248

Degenerate probe designed from conserved sequence is shown in bold.

The labelled probe hybridised strongly with the OXA-2-containing positive control strain *E. coli* K12, and less so with plasmid DNA from the *Acinetobacter* sp. transconjugant at low, medium and high stringencies (Figure 3.18). No signal was detected with plasmid DNA from strain 790; however, a faint signal was detected with the chromosomal DNA from both parent and cured colonies of strain 790.

Figure 3.18 Hybridisation of *bla*$_{ARI-2}$-positive strain 790 with ARI-NB degenerate probe



Low stringency wash

Lane

1	Strain 790 - chromosomal DNA
2	Strain 790/*Eco*R I
3	Cured strain 790
4	Cured strain 790/*Eco*R I
5	GeneRuler DNA ladder
6	BD413-2 transconjugant - plasmid DNA
7	BD413-2/*Eco*R I
8	*E. coli* K12 (OXA-2 producer)
9	*E. coli* K12/*Eco*R I
10	GeneRuler DNA ladder

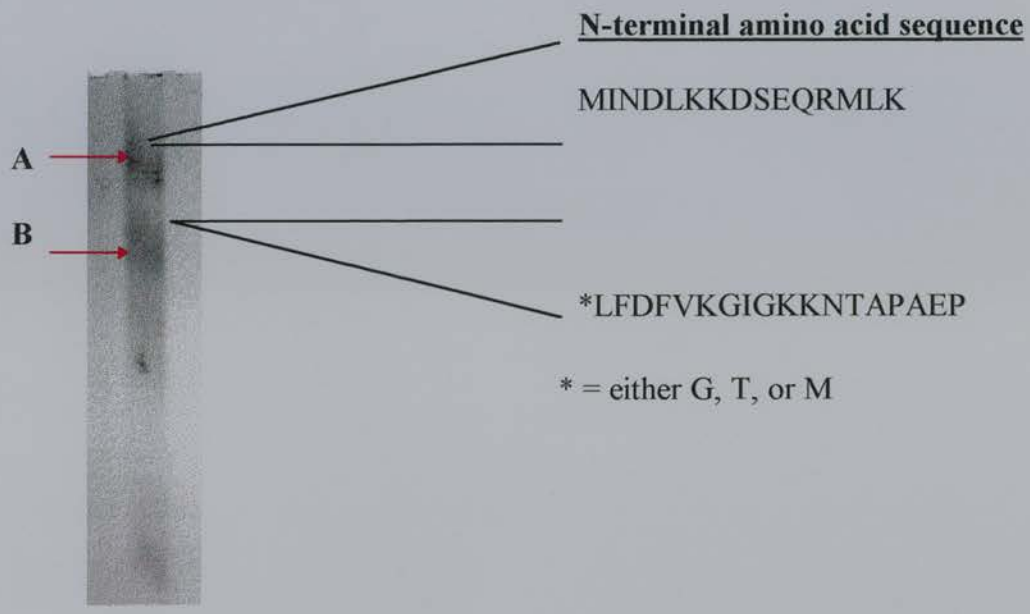
Faint signals with strain 790 in lanes 1 and 2, and with cured strain 790 in lane 3 are indicated by the red arrows.

3.13 N-terminal amino acid sequencing of the ARI-2 protein

Although the previous hybridisation results had suggested that the *bla*$_{ARI-2}$ gene encoded a molecular class D β-lactamase, it was proving difficult to obtain an initial sequence region that would enable the ARI-2 gene to be sequenced. Consequently, a different approach was undertaken in an attempt to solve this problem. The purification procedure with strain 788 had successfully separated ARI-2 from the higher molecular weight cephalosporinase. It was therefore decided to attempt to sequence the N-terminal end of the ARI-2 enzyme. The concentrated fractions that contained the ARI-2 β-lactamase (section 3.8) were subsequently subjected to electrophoresis on a native PAGE gel, and the proteins immobilised on a Problot membrane (sections 2.14.1 and 2.14.2). Two protein bands were identified as potentially being the ARI-2 β-lactamase which were both subjected to N-terminal amino acid sequencing (section 2.14.4).

A total of 15 amino acids were obtained for band A and 19 for band B (Figure 3.19). Both sequences were entered into a general protein sequence database for identification (BLASTP link at http://gc.bcm.tmc.edu:8088/search-launcher/launcher.html). There was no homology identified between both sequences and with any β-lactamases in the database. The sequence from band A however, shared 95% amino-acid homology with a ribosome binding protein which suggested that it was not from the ARI-2 protein band. The sequence from band B demonstrated no homology with any relevant proteins in the database. The possibility existed however, that this sequence could be a signal peptide of the ARI-2 β-lactamase. It was therefore decided to design a degenerate oligonucleotide from this sequence. This was subsequently labelled and used in a series of hybridisation reactions with the *bla*$_{ARI-2}$-positive strain 790.

Figure 3.19 Coomassie-stained native PAGE gel of fractions 29-34 from Superdex 75 gel filtration



3.13.1 Hybridisation with degenerate probe designed from N-terminal amino acid sequence of protein band B

The probe failed to hybridise to plasmid and chromosomal DNA from strain 790 even at low stringency. No signal was detected with the ARI-1 transconjugant.

3.13.2 Further purification and separation of the ARI-2 β-lactamase by RPHPLC

A final purification step was performed with fractions 29-34 in an attempt to remove the ARI-2 protein from other non-β-lactamase proteins that were also present. If this was successful, the protein could be digested with trypsin followed by sequencing of several of the resulting peptides in order to obtain some of the internal sequence of the protein. One of the peptide sequences (chosen at random) could then be used to design

a degenerate oligonucleotide, and subsequently used in conjunction with the ARI-2N degenerate primer to amplify the region between both sequences, providing that the chosen peptide sequence was sufficiently distant from the N-terminal itself.

Further purification of ARI-2 was performed by reversed-phase HPLC of fractions 29-34. The protein peaks were collected and subjected to electrophoresis on native PAGE gels, along with an aliquot of the ARI-2-containing fractions (as reference) which was stained with nitrocephin to identify the presence of the ARI-2 band. Unfortunately, the HPLC purification process renders enzymes inactive therefore, the gels containing the protein peaks could not be stained by this method. However, staining with Coomassie, and subsequently by the more sensitive silver staining method, failed to visualise protein bands from the collected peaks. The intensity of the reference lane was not strong, therefore it was possible that there was insufficient protein in the HPLC-purified samples for visualisation.

It was unfortunate that despite employing several different approaches, it was not possible to obtain the sequence of the ARI-2 gene in the time-scale of this work. However, the initial molecular studies of ARI-2-producing isolates suggested that this β-lactamase was a class D which appeared to be share some homology with the ARI-1 gene.

CHAPTER 4

DETERMINATION OF THE PRESENCE OF THE ARI-2 β-LACTAMASE IN CARBAPENEM-RESISTANT CLINICAL ISOLATES FROM HONG KONG, SINGAPORE, SPAIN AND TURKEY

4.1 Introduction

The discovery of the novel carbapenemase, ARI-2 in 17 clinical isolates from Argentina, raised the possibility that this β-lactamase may also be responsible for carbapenem resistance in clinical isolates from other areas worldwide. Consequently, a number of isolates collected from Hong Kong, Singapore, Spain and Turkey, that were reported to be resistant to the carbapenems, were investigated.

4.2 Clinical isolates

A total of 36 clinical isolates of *Acinetobacter* spp. were obtained from the 5 countries. Of these, 4 isolates were from the Hospital Clínic I Provincial in Barcelona, Spain, 6 were collected from The Prince of Wales Hospital in Hong Kong, 11 were from the KK Women's and Children's Hospital in Singapore, and the remaining 15 isolates were collected from Izmir, Turkey, the majority of which were from the Dokuz Eylul University Hospital. The isolates from Spain and Hong Kong were reported to have MIC values of imipenem of between 2 - >8 mg/L, and those from Turkey and Singapore were reported to be either of intermediate sensitivity or resistant by the Stokes method.

4.3 Antimicrobial susceptibility testing

The MIC values of the range of antibiotics tested are summarised in Table 4.1. All isolates were resistant to amoxycillin, co-amoxiclavulanic acid and cephaloridine, and the majority were resistant to both cefotaxime and ceftazidime (94% and 97.2% respectively). Among the 36 isolates, 83% were resistant to the aminoglycoside gentamicin, and 86% resistant to the fluoroquinolone ciprofloxacin. There was some variation observed in the MIC values of the carbapenems compared with those reported by the individual centres. A total of 56% of all isolates were resistant to imipenem, with the highest percentage of resistance demonstrated in the isolates from Singapore (82%). Overall, isolates demonstrated lower MIC values of meropenem

compared with imipenem, with the exception of those from Singapore, of which 100% had MIC values above the breakpoint for meropenem, compared with 82% above the breakpoint for imipenem. This trend was also observed with the isolates from Hong Kong, of which 67% were resistant to meropenem compared with 16% to imipenem.

Table 4.1 MIC values of *A. baumannii* clinical isolates from Spain, Hong Kong, Turkey and Singapore

Isolate	MIC values (mg/L)								
	Amox	Co-amox	Cld	Ctx	Caz	Imp	Mero	Cip	Gen
806 (Sp)	>128	128	>128	>256	>256	128	128	>16	64
807 (Sp)	>128	32	>128	16	8	0.25	0.25	8	32
808 (Sp)	>128	>128	>128	16	8	2	4	16	>128
809 (Sp)	>128	>128	>128	>128	64	8	4	8	64
811 (H)	>128	>128	128	>128	128	8	8	4	8
812 (H)	>128	>128	64	16	4	4	8	0.25	<0.12
813 (H)	>128	>128	64	16	8	4	8	0.25	<0.12
814 (H)	>128	>128	64	16	8	4	8	0.25	0.25
815 (H)	>128	32	>128	128	128	2	4	2	0.5
816 (H)	>128	64	>128	0.5	2	0.5	0.06	0.016	0.25
820 (T)	128	64	>128	0.5	8	0.5	0.12	0.03	0.5
821 (T)	>128	>128	>128	64	16	4	4	>16	16
822 (T)	>128	>128	>128	64	16	8	4	8	16
823 (T)	>128	>128	>128	64	16	8	4	>16	16
824 (T)	>128	>128	>128	64	16	8	4	8	16
825 (T)	>128	16	>128	>128	>128	0.25	0.5	>16	16
826 (T)	>128	>128	>128	32	16	8	4	8	16
827 (T)	>128	16	>128	>128	>128	0.25	0.5	>16	16
829 (T)	>128	>128	>128	32	16	8	4	8	16
830 (T)	>128	>128	>128	32	16	8	4	>16	16
869 (T)	>128	>128	>128	>128	>128	0.25	1	2	2
870 (T)	>128	>128	>128	>128	>128	8	8	8	8
871 (T)	>128	>128	>128	128	>128	8	8	2	>128

Table 4.1 continued

Isolate	Amox	Co-amox	Cld	Ctx	Caz	Imp	Mero	Cip	Gen
875 (T)	>128	>128	>128	>128	>128	0.25	2	8	2
876 (T)	>128	>128	>128	128	>128	8	4	16	>128
860 (S)	>128	>128	>128	>128	>128	8	64	>16	128
861 (S)	>128	>128	>128	>128	>128	8	64	>16	>128
862 (S)	>128	>128	>128	>128	>128	4	64	>16	>128
863 (S)	>128	>128	>128	>128	>128	16	64	>16	>128
864 (S)	>128	>128	>128	>128	>128	16	128	>16	>128
865 (S)	>128	>128	>128	>128	>128	8	64	>16	>128
866 (S)	>128	>128	>128	128	>128	8	64	>16	128
867 (S)	>128	>128	>128	>128	>128	8	32	>16	>128
868 (S)	>128	>128	>128	>128	>128	16	128	>16	>128
872 (S)	>128	>128	>128	>128	>128	4	64	>16	>128
873 (S)	>128	>128	>128	>128	>128	8	64	>16	>128

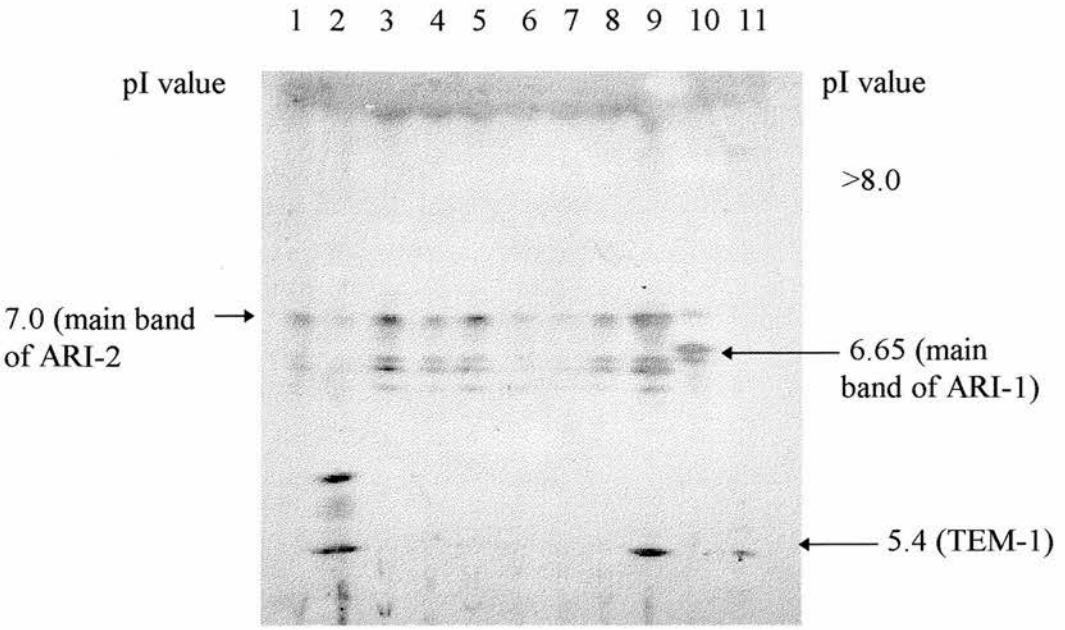
Abbreviations: Sp = Spain; H = Hong Kong; T = Turkey; S = Singapore
Amox; Amoxycillin, Co-amox; Co-amoxiclavulanic acid, Cld; Cephaloridine, Ctx; Cefotaxime, Caz; Ceftazidime, Imp; Imipenem, Mero; Meropenem, Cip; Ciprofloxacin, Gen; Gentamicin.
Recommended breakpoints (mg/L) (Working Party on Antibiotic Sensitivity Testing, 1991 and 1998a); Amox, Co-amox; 8.0, Cld, Caz; 2.0, Imp, Mero; 4.0, Gen, Ctx, Cip; 1.0

4.4 IEF analysis of imipenem-resistant isolates

Crude small-scale β-lactamase extracts were prepared from isolates that demonstrated an MIC value of imipenem of ≥ 4 mg/L. Table 4.2 lists the pI values of β-lactamases that were detected in these isolates.

Identical bands (pI 6.5 and 7.0) to those of the ARI-2 β-lactamase were visualised in crude extracts from 8 isolates, that comprised 6 isolates from Turkey, and 1 each from Spain and Hong Kong (Figure 4.1).

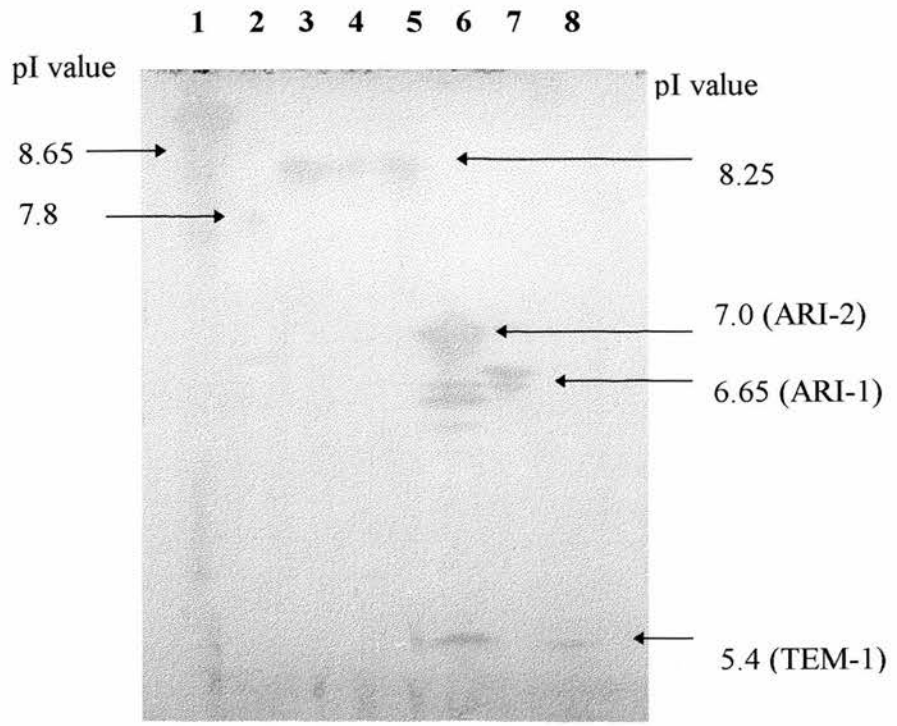
Figure 4.1 IEF gel of crude β-lactamase extracts of isolates from Spain, Hong Kong and Turkey demonstrating the presence of bands identical to those of ARI-2



Lane	Strain	Lane	Strain
1	809 (Spain)	7	829 (Turkey)
2	811 (Hong Kong)	8	830 (Turkey)
3	822 (Turkey)	9	790 (ARI-2 producer)
4	823 (Turkey)	10	6B 92 (ARI-1 producer)
5	824 (Turkey)	11	*E. coli* J62 (TEM-1 producer)
6	826 (Turkey)		

The majority of isolates also contained β-lactamase bands of >8.0 that resembled chromosomal cephalosporinases. A TEM-1-like β-lactamase was visualised in 4 isolates (1 from Hong Kong and 3 from Singapore). In addition, 5 isolates also demonstrated bands that did not resemble either the ARI-2-like β-lactamase, a cephalosporinase or the TEM-like β-lactamase (Figure 4.2). The pI value of the band visualised in isolate 806 from Spain was estimated as 8.65. A single isolate from Turkey (876) contained a β-lactamase band of pI 7.8, and 3 isolates (812, 813 and 814) from Hong Kong had an identical band of pI 8.25.

Figure 4.2 IEF gel of crude β-lactamase extracts of isolates from Spain, Hong Kong and Turkey demonstrating β-lactamases other than ARI-2



Lane	Strain and β-lactamase produced
1	Strain 806 (Spain) - pI 8.65
2	Strain 876 (Turkey) - pI 7.8
3	Strain 812 (Hong Kong) - pI 8.25
4	Strain 813 (Hong Kong) - pI 8.25
5	Strain 814 (Hong Kong) - pI 8.25
6	Strain 790 (Argentina) - pI 7.0 (ARI-2)
7	Strain 6B 92 (Edinburgh) - pI 6.65 (ARI-1)
8	*E. coli* J62-2 - TEM-1 producer

Table 4.2 pI values of β-lactamase bands present in imipenem-resistant isolates from Spain, Hong Kong, Turkey and Singapore

Isolate	pI values of β-lactamase bands
Spain	
806	8.65, >8.0
809	7.0, >8.0
Hong Kong	
811	5.4, 7.0, >8.0
812	8.25, >8.0
813	8.25, >8.0
814	8.25, >8.0
Turkey	
822	7.0, >8.0
823	7.0, >8.0
824	7.0, >8.0
826	7.0, >8.0
829	7.0, >8.0
830	7.0, >8.0
870	>8.0
871	>8.0
876	7.8
Singapore	
860	>8.0
861	>8.0
863	5.4, >8.0
864	>8.0
865	>8.0
866	>8.0
867	5.4, >8.0
868	>8.0
873	5.4, >8.0

4.4.1 Inhibitor overlays of IEF gels

Inhibitor overlays were applied to IEF gels containing the focused ARI-2-like β-lactamase and the unknown β-lactamases of pIs 8.65, 7.8 and 8.25. An identical inhibitor profile was obtained with the β-lactamases of main pI 7.0 in all 8 isolates (Table 4.3). The bands were not inhibited by EDTA, indicating that it was not a

metallo-enzyme. None of the bands were visible after overlays of both imipenem and BRL 42715, indicating that this enzyme was able to bind imipenem and that it had a serine residue at its active-site. These results were identical to those obtained with the 17 isolates from Argentina that contained the ARI-2 β-lactamase (section 3.3.2).

The β-lactamase of pI 8.25 in isolates 812, 813 and 814 was inhibited by imipenem, as was the enzyme of pI 7.8 in isolate 876 (Table 4.3). However, the activity of the β-lactamase of pI 8.65 in isolate 806 from Spain was not diminished by imipenem. All 3 β-lactamases were inhibited by BRL 42715, but not by EDTA.

In the majority of isolates, the cephalosporinase bands of pI >8.0 displayed a similar inhibitor profile to that of the cephalosporinases visualised in the Argentina isolates, in that activity was partially lost after the imipenem overlay. However, the cephalosporinases present in the isolates from Hong Kong were not affected by this overlay. Interestingly, the β-lactamase band of pI 8.9 in strain 806 (that also contained the β-lactamase of pI 8.65) was completely inhibited by imipenem.

The TEM-like enzyme identified in 4 isolates was inhibited by clavulanic acid and by BRL 42715, but not by EDTA or imipenem. This was in agreement with the inhibitor overlay results obtained with both the TEM-1 enzyme in the isolates from Argentina, and with a TEM-1 producer (*E. coli* J62-2) (section 3.3.2).

Table 4.3 Inhibitor profiles of β-lactamases by IEF inhibitor overlays

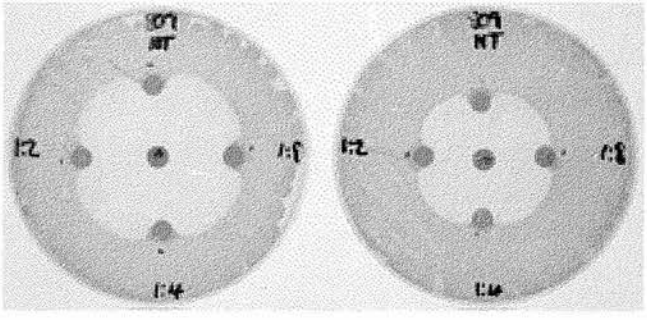
Isolate	β-lactamase (pI)	BRL 42715 (100μM)	EDTA (1mM)	Imipenem (100μM)
809, 811, 822, 823, 824, 826, 829, 830	7.0	+	-	-
806	8.65	+	-	-
	8.9	+	-	+
812, 813, 814	8.25	+	-	+
876	7.8	+	-	+

4.5 Microbiological assays of crude β-lactamase extracts

To determine whether β-lactamase-mediated inactivation of the carbapenems occurred with imipenem-resistant isolates that produced the β-lactamases of pIs 7.0, 7.8, 8.25 and 8.65, crude β-lactamase extracts were subjected to the microbiological assay. All isolates that produced the ARI-2-like β-lactamase demonstrated imipenem inactivation by this method (Figure 4.3). Meropenem inactivation was also detected but to a lesser extent (Figure 4.3), which was also demonstrated with the ARI-2-producing strains from Argentina (Figure 3.4). Inactivation of both carbapenems was demonstrated with isolate 806 that produced the β-lactamase of pI 8.65 (Figure 4.4), despite the fact that an overlay of imipenem had failed to eliminate the activity of this enzyme. Isolates 812, 813 and 814 which produced the β-lactamase of pI 8.25 also demonstrated inactivation of both carbapenems by this method (Figure 4.4). The crude extract of isolate 876 which produced the enzyme of pI 7.8 failed to inactivate meropenem. In addition, imipenem inactivation by this extract was relatively weak (Figure 4.4).

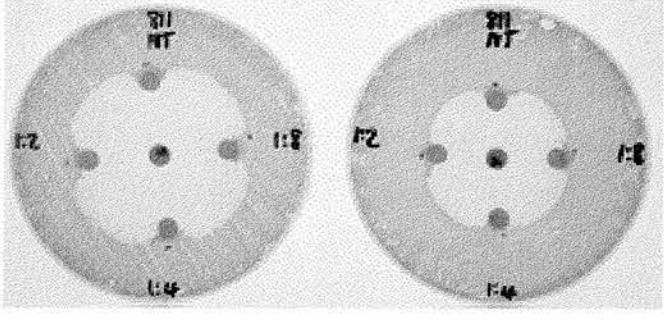
Figure 4.3 Microbiological assays of crude extracts from isolates producing ARI-2-like β-lactamase of pI 7.0

Imipenem Meropenem



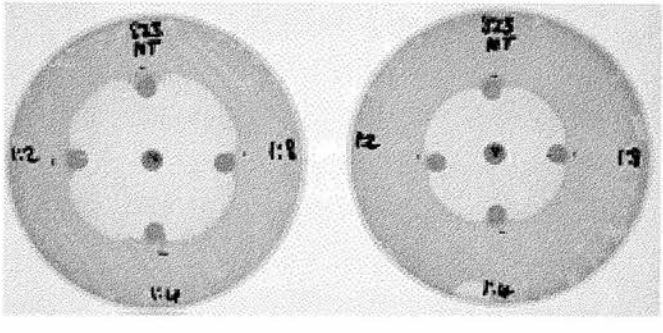
Strain 809 (Spain)

Imipenem Meropenem



Strain 811 from Hong Kong

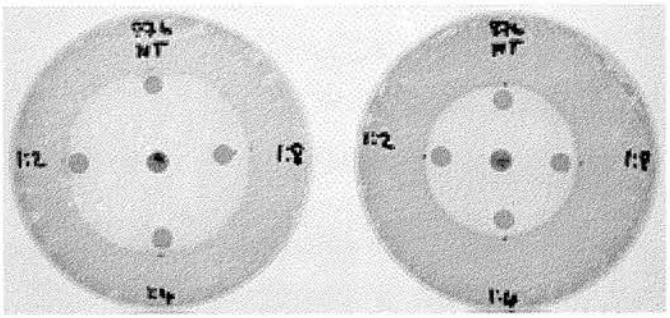
Imipenem Meropenem



Representative strain 823 from Turkey

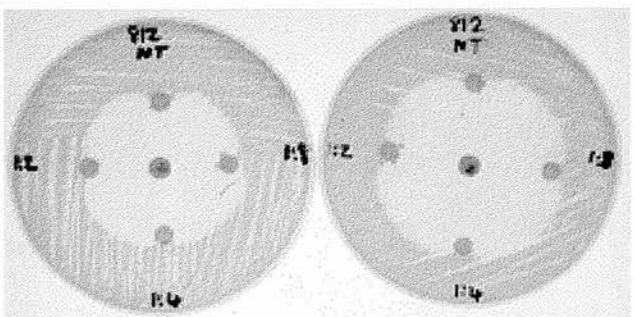
Figure 4.4 Microbiological assays of crude extracts from isolates producing β-lactamases of pI values 7.8, 8.25 and 8.65

Imipenem Meropenem



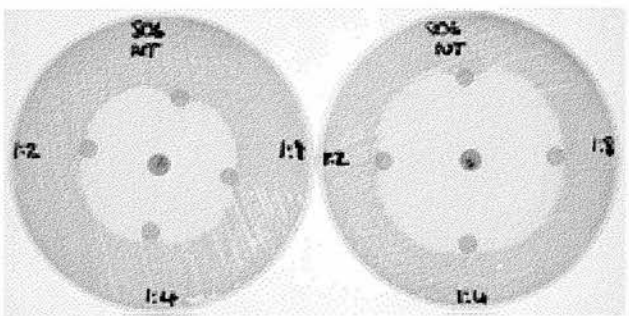
Strain 876 (pI 7.8) from Turkey

Meropenem Imipenem



Representative strain 812 (pI 8.25) from Hong Kong

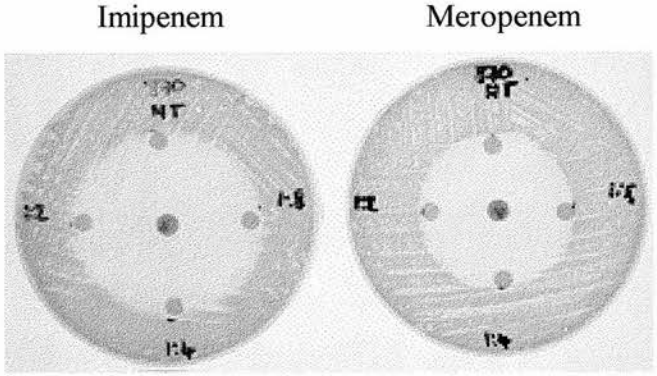
Meropenem Imipenem



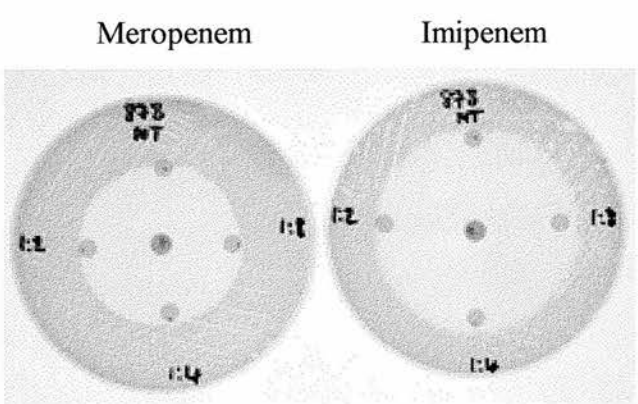
Strain 806 (pI 8.65) from Spain

Crude β-lactamase extracts from strains 870 and 873 were also subjected to the microbiological assay to determine whether the activity of either a cephalosporinase alone (strain 870 from Turkey), or in combination with TEM-1 (strain 873 from Singapore) resulted in carbapenem inactivation. However, the extracts failed to inactivate both imipenem and meropenem (Figure 4.5), indicating that β-lactamase activity was not responsible for carbapenem resistance detected in these strains.

Figure 4.5 Microbiological assays of crude extracts from isolates producing cephalosporinase and TEM-1 β-lactamase



Strain 870 (Turkey) producing cephalosporinase only



Strain 873 (Singapore) producing both TEM-1 and cephalosporinase

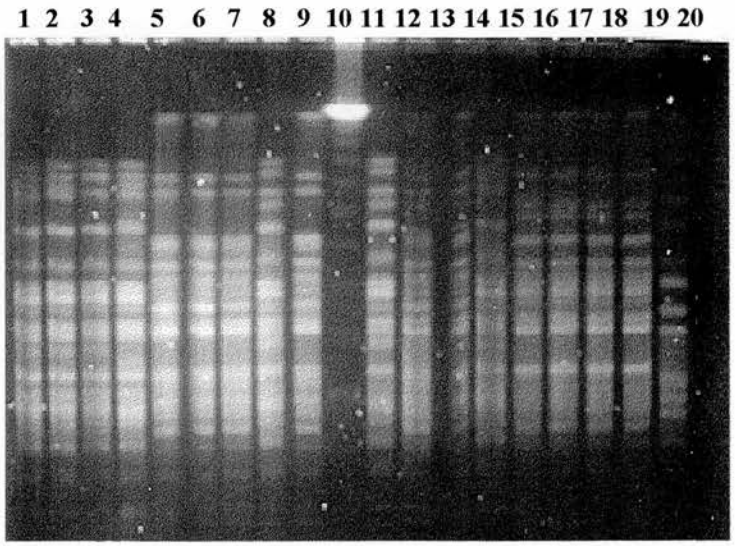
4.6 Analysis of isolates by PFGE

From the preliminary investigations of these carbapenem-resistant isolates, it appeared that the ARI-2 β-lactamase was not confined to Buenos Aires, but was also present in isolates from Turkey, Spain and Hong Kong. To determine whether its dissemination within a particular area was as a result of clonal spread or from transfer of the bla_{ARI-2} gene between genetically unrelated isolates, DNA restriction fragment length polymorphism (RFLP) patterns of all ARI-2-producing isolates were analysed.

The RFLP patterns are illustrated in Figures 4.6 and 4.7. Patterns were compared visually in addition to being analysed by the GelCompar computer system, which processes digitised images of the RFLP patterns. Data were normalised by the alignment of molecular size standards that were run in 2 separate lanes in each gel. In addition, the imipenem-sensitive strain, 789 was also included in each gel. The program generated a matrix of similarity coefficients between all possible pairs of strains, leading in turn to a dendrogram (Figure 4.8) in which all patterns were placed in a horizontal tree with branching dictated by the similarity coefficients. Results were expressed in terms of percentage correlation values. Clusters of identical or closely related strains were labelled based on a correlation value cut-off of 80%.

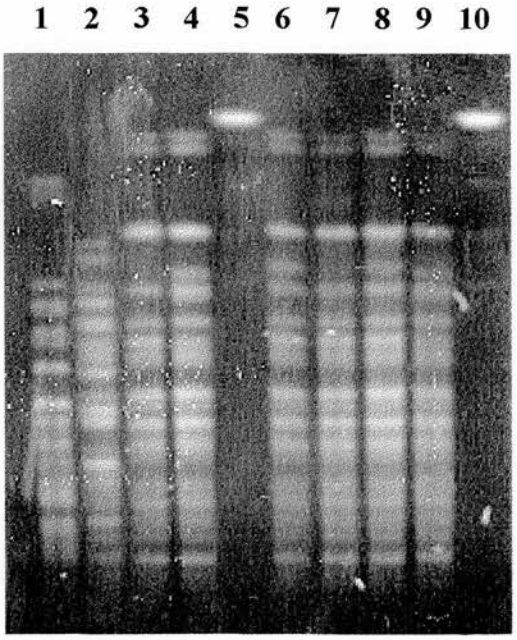
In general, the clusters produced by the GelCompar program correlated well with visual comparison of the RFLP patterns. Five main clusters were identified, A, B, C, D and E. Clusters A, B, C and E included isolates from Argentina (Table 4.4). Cluster A comprised those isolated from the CEMIC teaching hospital between 1993 and 1994, which shared similarities of between 90 and 97%. Cluster B was also indigenous to this teaching hospital and comprised 4 isolates with identical RFLP patterns that were collected within a 2 month period in 1998. A similarity value of 60% was obtained when the patterns of clusters A and B were compared.

Figure 4.6 PFGE of RFLP patterns of ARI-2-producing isolates from Buenos Aires, Argentina



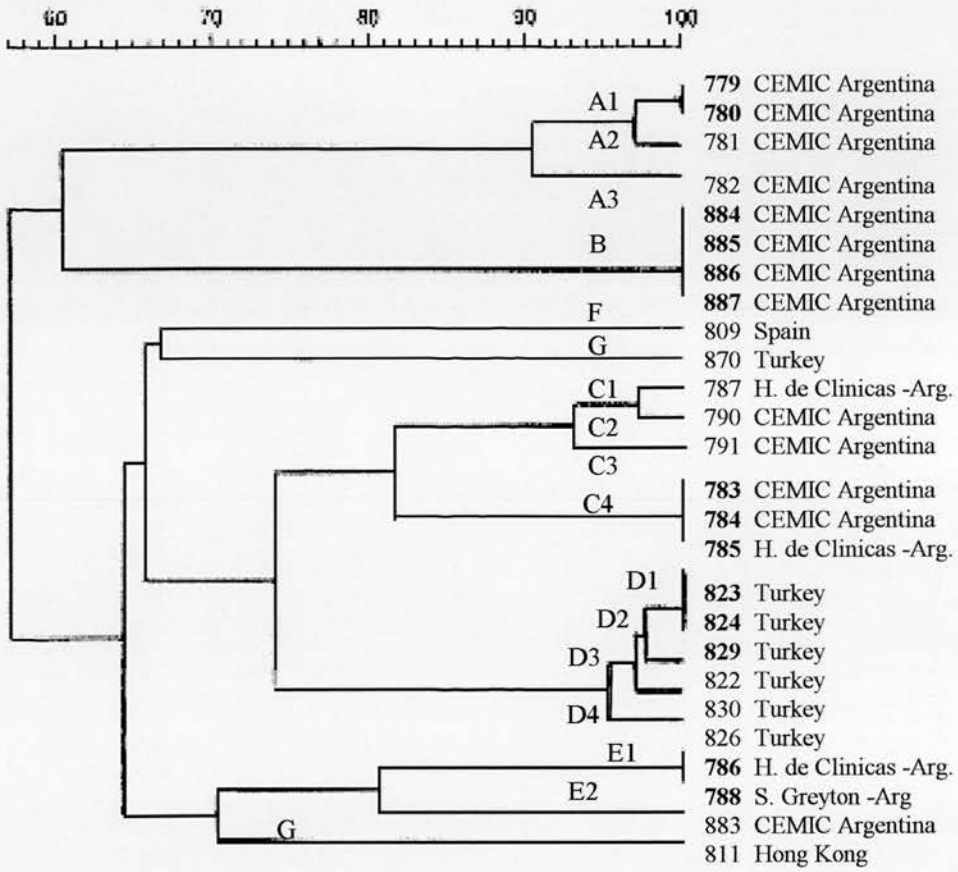
Lane	Isolate
1	779
2	780
3	781
4	782
5	783
6	784
7	785
8	786
9	787
10	λ ladder (range 48.5-970 kbp)
11	788
12	790
13	791
14	883
15	884
16	885
17	886
18	887
19	789 (imipenem-sensitive isolate)
20	λ ladder

Figure 4.7 PFGE of RFLP patterns of ARI-2-producing isolates from Turkey, Spain and Hong Kong



Lane	Isolate
1	809 (Spain)
2	811 (Hong Kong)
3	822 (Turkey)
4	823 (Turkey)
5	λ DNA ladder (range 48.5-970 kbp)
6	824 (Turkey)
7	826 (Turkey)
8	829 (Turkey)
9	830 (Turkey)
10	λ DNA

Figure 4.8 Gel-Compar-generated UPGMA clustering dendrogram of RFLP patterns of ARI-2-producing isolates



Groups of isolates in bold share identical RFLP patterns

Cluster C included isolates from 2 teaching hospitals in Buenos Aires, and which shared between 93-97% similarity values. Isolates 784 (from CEMIC) and 785 (from Hospital de Clinicas) had identical RFLP patterns. All of the isolates from the Dokuz Eylul hospital in Turkey, with the exception of 870, the clinical details of which were unknown, comprised cluster D. Similarity values of between 97% and 100% were obtained for these isolates. This suggested that clonal spread of carbapenem resistance had occurred in this particular area.

Table 4.4 Geographical origins and designated clusters of ARI-2-producing isolates

Isolate number	Geographical origin	Date of isolation	Hospital/ward	Source	RFLP pattern
779	Buenos Aires, Argentina	02/10/93	C.E.M.I.C	Cath. site	A1
780	Buenos Aires	13/03/94	C.E.M.I.C	Cath. site	A1
781	Buenos Aires	02/05/94	C.E.M.I.C	BAL	A2
782	Buenos Aires	03/11/94	C.E.M.I.C	BAL	A3
783	Buenos Aires	21/07/95	C.E.M.I.C	BAL	C4
784	Buenos Aires	22/07/94	C.E.M.I.C	Cath. site	C4
785	Buenos Aires	1994	H. de Clinicas	BAL	C4
786	Buenos Aires	1994	H. de Clinicas	BAL	E1
787	Buenos Aires	1994	H. de Clinicas	Soft tissue	C1
788	Buenos Aires	21/08/94	Sanatorio Greyton	Urine	E1
790	Buenos Aires	07/10/95	C.E.M.I.C	BAL	C2
791	Buenos Aires	16/10/95	C.E.M.I.C	BAL	C3
883	Buenos Aires	1995	C.E.M.I.C	BAL	E2
884	Buenos Aires	7/98-8/98	C.E.M.I.C	BAL	B
885	Buenos Aires	7/98-8/98	C.E.M.I.C	BAL	B
886	Buenos Aires	7/98-8/98	C.E.M.I.C	BAL	B
887	Buenos Aires	7/98-8/98	C.E.M.I.C	Cath. site & blood culture	B
822	Izmir, Turkey	6/96-4/97	Dokuz Eylul/ ITU	Tracheal asp.	D2
823	Izmir	6/96-4/97	D. Eylul/ Neurosurgery	Wound	D1
824	Izmir	6/96-4/97	D. Eylul/ ITU	Tracheal asp.	D1
826	Izmir	6/96-4/97	D. Eylul/ Thoracic surgery	Tracheal asp.	D4

Table 4.4 continued

Isolate number	Geographical origin	Date of isolation	Hospital/ward	Source	RFLP pattern
829	Izmir	6/96-4/97	D. Eylul/Thoracic surgery	Tracheal asp.	D1
830	Izmir	6/96-4/97	D. Eylul/ Neurosurgery	Spinal fluid	D3
870	Unknown	Unknown	Unknown	Unknown	H
809	Barcelona, Spain	Unknown	Unknown	Unknown	F
811	Hong Kong	01/01/96	Prince of Wales Hospital	Deep wound	G

Abbreviations: C.E.M.I.C; Centro de Educación Médica e Investigaciones Clínicas, H. de Clinicas; Hospital de Clinicas teaching hospital, Sanatorio Greyton; geriatric hospital, D. Eylul; Dokuz Eylul University Hospital, Cath. Site; catheter site, BAL; bronchial alveolar lavage, Tracheal asp; tracheal aspirate

The isolates grouped in cluster E originated from all 3 hospitals in Buenos Aires. As with cluster C, this group included isolates (786 and 788) from 2 different hospitals, that had identical RFLP patterns.

The RFLP patterns of the single isolates from Spain and from Hong Kong differed markedly from each other and from the main clusters.

The appearance of 4 different carbapenem-resistant strains within CEMIC, and 2 within the Hospital de Clinicas, strongly suggested that horizontal transfer of the ARI-2 gene had occurred between these strains. Interestingly, strains 883 (cluster E2) and 884 (cluster B1) which were identified as being unrelated by GelCompar and visual comparison of RFLPs, were both isolated from the same patient from CEMIC, the former in 1995 and the latter in 1998.

CHAPTER 5

THE DIFFERENTIAL ACTIVITY OF FLUOROQUINOLONES AGAINST MULTI-RESISTANT CLINICAL ISOLATES OF *ACINETOBACTER* SPECIES

5.1 Introduction

For over a decade, the fluoroquinolones have been used successfully in the treatment and management of severe nosocomial infections caused by mullti-resistant bacteria, including *Acinetobacter baumannii*. However, fluoroquinolone resistance has emerged in this species (Joly-Guillou and Bergogne-Bérézin, 1992), which is of particular concern given that so many antibiotics, including imipenem, are no longer guaranteed effective in treating many *A. baumannii* infections.

Fluoroquinolones exert their antibacterial effect by inhibition of 2 bacterial topoisomerase enzymes namely DNA gyrase (topoisomerase II) and topoisomerase IV, that are responsible for the complex reaction of DNA supercoiling in prokaryotes (Drlica and Zhao, 1997). The key event in quinolone action involves formation of a quinolone-enzyme-DNA complex. This is followed by a rapid, reversible inhibition of DNA synthesis, and at higher drug concentrations, cell death follows as a result of the release of double-strand DNA breaks from the complex (Drlica and Zhao, 1997). DNA gyrase is the primary target of the fluoroquinolones in Gram-negative bacteria (Hooper, 1999).

Resistance to this class of antibiotic is mediated by mutational changes in the genes encoding the target enzymes, which occur within a region extending between amino acids 67 and 106, called the quinolone resistance-determining region (QRDR) (Piddock, 1999). Within *gyrA* of *E. coli*, mutations of serine 83 and aspartic acid 87 result in the greatest reduction in susceptibility (Yoshida *et al.*, 1990). Mutations in the GyrB sub-unit of DNA gyrase may also contribute to decreased susceptibility but they are less common (Nakamura *et al.*, 1989). In addition, other mechanisms that may be involved in fluoroquinolone resistance include a decrease in outer membrane proteins (Hooper *et al.*, 1992) or alterations in outer membrane lipopolysaccharides (Hirai *et al.* 1986), both of which result in a reduction in the intracellular accumulation of the antibiotic. Active efflux of quinolones at the inner membrane is

also a likely contributor of resistance in both Gram-negative and Gram-positive bacteria (Nikaido, 1996; Piddock, 1999).

Although there have been several studies reporting resistance rates of *Acinetobacter* species against the fluoroquinolones (Fass *et al.*, 1996; Barry and Fuchs, 1997; Balakrishnan *et al.*, 1999), there is little evidence of the efficacy of these antibiotics against clinical isolates of imipenem-resistant isolates. The aim of this study therefore, was to ascertain the activity of seven fluoroquinolones against multi-resistant clinical isolates collected from Argentina, Spain, Hong Kong and Turkey, which represent some of the most resistant *Acinetobacter* strains world wide, and several of which produce the novel carbapenemase ARI-2.

5.2 Source of clinical isolates

A total of 39 clinical isolates from Argentina, Turkey, Spain and Hong Kong were tested (Table 5.1). The majority of these were multi-resistant with 58.9% demonstrating imipenem resistance, of which 86.9% produced the ARI-2 β-lactamase.

Table 5.1 Source of clinical isolates

Country of origin	Number of isolates
Turkey	16
Argentina	13
Hong Kong	6
Spain	4

5.3 Antimicrobial susceptibility testing

MIC values (range of 0.004-128 mg/L) were determined of all quinolones, with the exception of levofloxacin, which was tested by the Stokes method.

Table 5.2 MIC values of fluoroquinolones against multi-resistant clinical isolates of *A. baumannii*

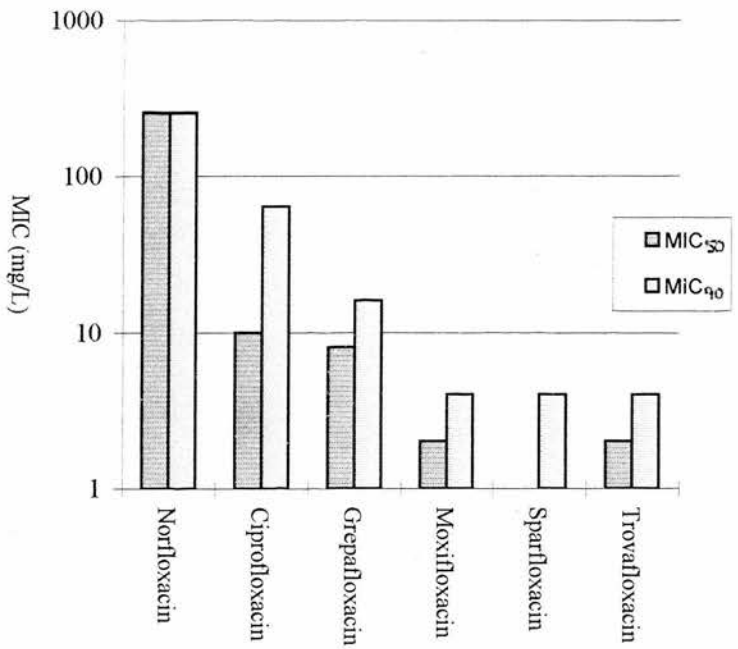
Isolate	MIC values (mg/L)					
	Cip	Moxi	Spar	Grepa	Trova	Norflox
779	64	2	2	16	2	256
780	32	2	2	16	2	256
781	32	2	2	16	2	256
782	32	2	1	8	2	256
783	32	2	2	8	2	256
784	32	4	2	8	2	256
785	16	2	1	8	2	256
786	32	2	2	8	2	256
787	32	2	1	8	2	256
788	32	2	2	8	2	256
789	16	8	4	2	2	256
790	16	2	1	2	2	256
791	16	2	1	2	2	256
806	32	2	4	16	2	256
807	16	2	0.5	2	1	128
808	4	2	1	0.5	0.12	64
809	2	0.12	0.25	0.25	0.12	32
811	8	2	0.25	0.12	0.032	64
812	2	0.25	0.25	0.12	0.016	64
813	0.064	0.008	0.004	0.008	0.004	2
814	0.064	0.008	0.004	0.008	0.004	2
815	2	0.12	0.25	0.12	0.12	16
816	0.008	0.032	0.016	0.008	0.016	0.5
820	0.008	0.032	0.032	0.016	0.016	0.25
821	16	2	1	2	2	256
822	4	0.25	0.12	0.25	0.5	64
823	16	2	2	4	2	256
824	2	0.12	0.12	0.12	0.12	32
825	32	2	2	32	4	256
827	32	2	4	16	2	256
829	2	0.12	0.25	0.12	0.12	16
830	16	2	2	16	2	256
833	32	4	4	32	2	256
834	128	4	16	8	8	256
836	128	4	8	8	8	256
837	128	4	2	8	4	256
838	16	0.5	1	8	2	1
839	0.064	0.12	0.064	0.064	0.032	0.5
850	2	0.12	0.12	0.12	0.12	1

Abbreviations: Cip; Ciprofloxacin, Moxi; Moxifloxacin, Spar; Sparfloxacin, Grepa; Grepafloxacin, Trova; Trovafloxacin, Norflox; Norfloxacin. **Breakpoint**; 1.0 mg/L

Table 5.3 MIC ranges, MIC_{50} and MIC_{90} values

Antibiotic	MIC range (mg/L)		MIC_{50}	MIC_{90}	%
	Low	High	(mg/L)	(mg/L)	Resistance
Ciprofloxacin	0.008	128	16	64	89.7
Norfloxacin	0.25	256	256	256	87.2
Grepafloxacin	0.008	32	8	16	64.1
Moxifloxacin	0.008	8	2	4	66.7
Sparfloxacin	0.004	16	1	4	43.6
Trovafloxacin	0.004	8	2	4	61.5
Levofloxacin	N/A	N/A	N/A	N/A	59

Figure 5.1 Graph representation of MIC_{50} and MIC_{90} values



The isolates varied in their sensitivity to the quinolones tested (Table 5.3 and Figure 5.1). Significant resistance was observed to ciprofloxacin and norfloxacin. The highest MIC_{50} and MIC_{90} values were demonstrated for norfloxacin (256 mg/L for

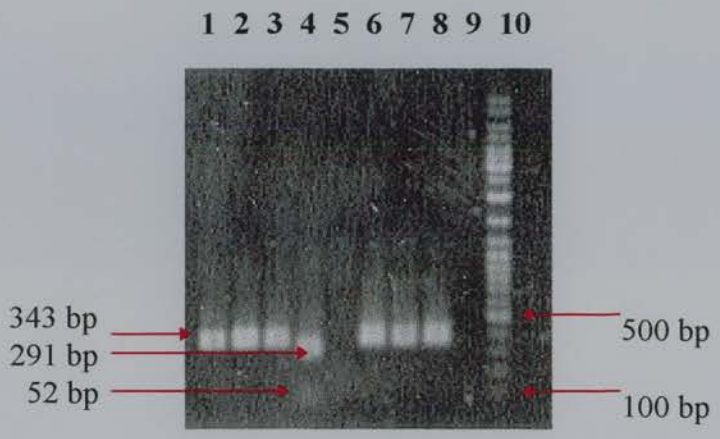
both). Comparable values were detected for ciprofloxacin and grepafloxacin. The most active of the fluoroquinolones were sparfloxacin, trovafloxacin and moxifloxacin, with the lowest MIC_{50} and MIC_{90} values detected for sparfloxacin (1.0 mg/L and 4 mg/L respectively). Overall, the highest resistance levels were demonstrated by the isolates from Argentina, of which 62% were resistant to all the quinolones tested. No correlation was observed between carbapenem and fluoroquinolone resistance.

5.4 Investigation of ciprofloxacin-resistant isolates by PCR amplification of the QRDR of *gyrA*

PCR amplification of the QRDR of the *gyrA* gene was performed with primers designed from conserved amino acid sequence motifs identified in several diverse *gyrA* genes (section 2.20). PCR products were subsequently digested with *Hinf* I (section 2.18). The conserved Asp-Ser residues at positions equivalent to 82 and 83 for *E. coli* form a *Hinf* I restriction site, which is subsequently abolished in isolates carrying a mutation at codon 83, as indicated by no digestion of the PCR product.

Amplification of the QRDR of the *gyrA* gene yielded a PCR product of 343 bp in all isolates (Figure 5.2). *Hinf* I restriction of the PCR products from the isolates with ciprofloxacin MIC values of 0.008-0.064 mg/L yielded DNA fragments of 291 bp and 52 bp (Figure 5.2), indicating that the *Hinf* I site was intact. Digestion of the PCR products from isolates with ciprofloxacin MIC values of $\geq$ 2 mg/L failed to produce restriction fragments, indicating the loss of the *Hinf* I restriction site at the codons for amino acids 82-83 (Figure 5.2).

Figure 5.2 *Hinf* I restriction of PCR-amplified *gyrA* QRDR of ciprofloxacin-sensitive and ciprofloxacin-resistant *A. baumannii* clinical isolates



Lane	Isolate	Ciprofloxacin MIC (mg/L)
1	779	64
2	808	4
3	811	8
4	813	0.064
5	820	0.008 (did not amplify)
6	821	16
7	825	32
8	834	128
9	Empty lane	
10	GeneRuler DNA fragments	

Sequencing of the PCR products from representative ciprofloxacin-resistant isolates confirmed the presence of a change at Ser-83 to Leu (C ⟶ T transversion from codon TCA) in all isolates with a MIC value of ≥ 2 mg/L (Figure 5.3). No other amino acid changes were identified. The ciprofloxacin-sensitive isolates with the intact *Hinf* I site retained the TCA codon at this position.

Figure 5.3 Comparison of partial DNA sequences (containing the Ser-83 region) of ciprofloxacin-resistant clinical isolates and the wild-type *A. baumannii gyrA* gene

	76									85
Cip-R isolates	AAA	TAT	CAC	CCG	CAT	GGT	GAC	T**T**A	GCT	GTT
	Lys	Tyr	His	Pro	His	Gly	Asp	**Leu**	Ala	Val
A. baumanii gyrA	AAA	TAT	CAC	CCG	CAT	GGT	GAC	TCA	GCT	GTT
	Lys	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Val

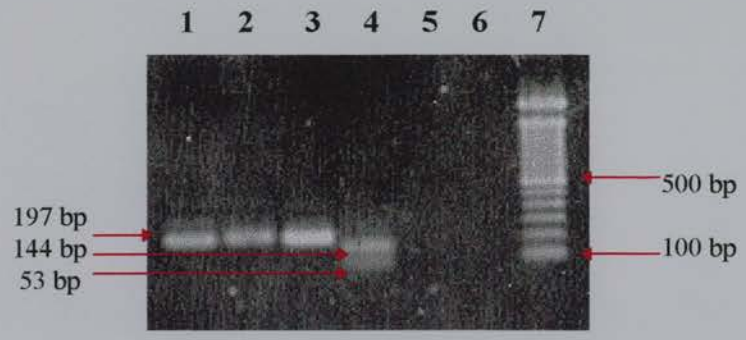
Mutation at Ser-83 in ciprofloxacin-resistant isolates is indicated in bold.
The deduced amino acid sequences are given below each nucleotide sequence

5.5 PCR amplification of the QRDR of *parC* of ciprofloxacin-resistant clinical isolates

Investigation of the QRDR of *gyrA* of clinical isolates had revealed a Ser-83 substitution in isolates with a ciprofloxacin MIC value of 2-128 mg/L. To investigate whether a mutation in the *parC* gene could be involved in the variation observed in the MIC values, PCR amplification of this region was performed with *parC*-specific primers (Tables 2.8 and 2.9) which produces a product of 197 bp in size. PCR products were subsequently digested with *Hinf* I (section 2.18). A *Hinf* I restriction site is present within the QRDR region at the codons for amino acids Asp-Ser 79-80, which is abolished in isolates carrying a mutation at codon 80.

PCR amplification yielded a product of 197 bp in all isolates (Figure 5.4). *Hinf* I restriction of PCR products from the isolates with ciprofloxacin MIC values of 2-8 mg/L produced 2 DNA fragments of 144 bp and 53 bp (Figure 5.4), indicating that the *Hinf* I site was intact. Restriction of the PCR products from the majority of isolates with a ciprofloxacin MIC value of ≥ 16 mg/L failed to produce these restriction fragments, indicating that the *Hinf* I site had been lost. However, restriction of PCR products from four isolates with MIC values of 16 mg/L resulted in visualisation of fragments of 144 bp and 53 bp, indicating that the *Hinf* I restriction site had not been lost in these particular isolates (Table 5.4).

Figure 5.4 *Hinf* I restriction of PCR-amplified *parC* of ciprofloxacin-resistant clinical isolates



Lane	Isolate
1	Non-restricted PCR product of isolate 779 (Cip MIC value of 64 mg/L)
2	*Hinf* I-restricted PCR product of 779
3	Non-restricted PCR product of isolate 811 (Cip MIC value of 8 mg/L)
4	*Hinf* I-restricted PCR product of isolate 811
5	Negative control (no primers)
6	Negative control (no DNA)
7	100 bp DNA ladder

Sequencing of PCR products detected a change at Ser-80 to Leu (C → T transversion from codon TCG) in the majority of high-level ciprofloxacin-resistant isolates (≥ 16 mg/L) (Figure 5.5). However, sequencing of the product from isolate 830 (MIC of 16 mg/L) which had retained the *Hinf* I restriction site revealed that this change was not present, but a Glu-84 to Lys change had occurred (Figure 5.5). No *parC* mutation was found in the absence of the *gyrA* mutation.

Figure 5.5 Comparison of partial DNA sequences of the QRDR of the *parC* gene of high-level ciprofloxacin-resistant clinical isolates

Isolate		**Cip MIC (mg/L)**
	80 **84**	
A. baumannii parC	GGT GAC TCG GCA TGT TAT GAA GCC Gly Asp Ser Ala Cys Tyr Glu Ala	
836	GGT GAC TCG GN- TGT TAT GAA GCC Ser	128
779	GGT GAC T**T**G GCA TGT TAT GAA GCC **Leu**	64
780	GGT GAC T**T**G GCA TGT TAT GAA GCC **Leu**	32
825	GGT GAC T**T**G GN- TGT TAT GAA GCC **Leu**	32
830	GGT GAC TCG GCA TGT TAT **A**AA GCC Ser **Lys**	16

Mutation at Ser-80 is indicated in bold. Mutation at Glu-84 to Lys in isolate 830 also indicated in bold.

The deduced amino acid sequences are given below the nucleotide sequences.

Table 5.4 *Hinf* I restriction of *parC* QRDR of high-level ciprofloxacin-resistant clinical isolates

Isolate	Ciprofloxacin MIC (mg/L)	*parC* Ser-80 mutation
834	128	+
836	128	+
837	128	+
779	64	+
780	32	+
781	32	+
782	32	+
783	32	+
784	32	+
786	32	+
787	32	+
788	32	+
806	32	+
825	32	+
833	32	+
785	16	+
789	16	+
790	16	+
791	16	+
807	16	+
821	16	-
823	16	-
830	16	-
838	16	-

+ = mutation present
- = mutation not present
The *gyrA* Ser-83 to Leu mutation was present in all isolates

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CHAPTER 6

A STUDY OF THE EFFICACY OF SULBACTAM AGAINST CARBAPENEM-RESISTANT CLINICAL ISOLATES OF *ACINETOBACTER* SPECIES

6.1 Introduction

The penicillanic acid sulphone derivative, sulbactam was first reported by Pfizer's chemists in 1978 and was shown to have potent inhibitory activity, particularly against molecular class A β-lactamases, although it also demonstrated a greater affinity than clavulanic acid for class C enzymes (English *et al.*, 1978). Sulbactam is most frequently used in combination with ampicillin (2:1 ratio ampicillin), which increases the spectrum of this β-lactam to include β-lactamase-producing strains of among others, *Klebsiella* spp., *Proteus* spp., *S. aureus* and *E. coli* (Sutherland, 1995). This combination has been reported to be effective against both imipenem-sensitive (Pandey *et al.*, 1998; Faas *et al.*, 1996) and imipenem-resistant *A. baumannii* isolates (Jiménez-Mejías *et al.*, 1997).

Although sulbactam displays only modest antimicrobial activity in its own right, its specific *in vitro* activity against *Acinetobacter* spp. has been documented (Go *et al.*, 1994; Corbella *et al.*, 1998). This has been attributed to the selective affinity of sulbactam for PBPs in particular, PBP2 (Urban *et al.*, 1995). There have been reports of multi-resistant *Acinetobacter* spp. that are also resistant to imipenem but remain susceptible to sulbactam (Go *et al.*, 1994; Corbella *et al.*, 1998), which has raised the possibility of the use of this inhibitor in treating infections caused by these bacteria. However, resistance has already been observed (Wood and Reboli, 1993; Traub and Spohr, 1989; Fernandez-Viladrich *et al*, 1999). In addition, reports have advised against the use of sulbactam as monotherapy for *A. baumannii* infections because of its poor bactericidal activity against many strains (Aubert *et al.*, 1996).

Several studies have reported the effectiveness of other sulbactam combinations. Cefoperazone plus sulbactam is available in a number of countries, including Japan (Sutherland, 1995), and this combination along with imipenem has been used in Turkey to treat *A. baumannii* infections (Akalm *et al.*, 1999). Other combinations include sulbactam with ticarcillin (Joly-Guillou *et al.*, 1995) or with cefpirome (Aubert *et al.*, 1996). The use of sulbactam in combination with the carbapenems has

not been documented; however, one study has reported a superior bacteriostatic and cidal effect with this combination against imipenem-sensitive isolates (Aubert *et al.*, 1996).

The aim of this study was therefore to evaluate the efficacy of sulbactam alone and in combination with imipenem against imipenem-sensitive and imipenem-resistant clinical isolates of *Acinetobacter* spp. in order to establish whether carbapenem/β-lactamase inhibitor therapy could provide an alternative means of successfully treating infections caused by these bacteria.

6.2 Bacterial isolates

A total of 35 clinical isolates of *Acinetobacter* spp. were analysed in this study (Table 6.1). Of these, 13 isolates were from Buenos Aires, Argentina, of which 92% were imipenem-resistant and produced the ARI-2 β-lactamase. Another 13 isolates originated from 2 hospitals within Scotland, the majority of which were imipenem-sensitive. The other 9 isolates comprised 4 from Spain, of which 2 were imipenem-resistant (including 1 ARI-2-producing isolate), and 5 from Hong Kong, of which 4 were imipenem-resistant (including 3 that produced an unknown β-lactamase of pI 8.25, and 1 ARI-2-producing isolate).

The majority of isolates were identified as *A. baumannii*, with the exception of 7 that comprised 5 *A. junii* and 2 *A. haemolyticus* isolates (Table 6.1).

Table 6.1 Details of clinical isolates of *Acinetobacter* spp.

Isolate number	Identification	Origin
779	*A. baumannii*	Buenos Aires, Argentina
780	*A. baumannii*	Buenos Aires, Argentina
781	*A. baumannii*	Buenos Aires, Argentina
782	*A. baumannii*	Buenos Aires, Argentina
783	*A. baumannii*	Buenos Aires, Argentina
784	*A. baumannii*	Buenos Aires, Argentina
785	*A. baumannii*	Buenos Aires, Argentina
786	*A. baumannii*	Buenos Aires, Argentina
787	*A. junii*	Buenos Aires, Argentina
788	*A. baumannii*	Buenos Aires, Argentina
789	*A. baumannii*	Buenos Aires, Argentina
790	*A. baumannii*	Buenos Aires, Argentina
791	*A. baumannii*	Buenos Aires, Argentina
792	*A. baumannii*	RIE, Edinburgh
803	*A. baumannii*	RIE, Edinburgh
804	*A. junii*	RIE, Edinburgh
806	*A. baumannii*	Barcelona, Spain
807	*A. baumannii*	Barcelona, Spain
808	*A. baumannii*	Barcelona, Spain
809	*A. baumannii*	Barcelona, Spain
811	*A. baumannii*	Hong Kong
812	*A. baumannii*	Hong Kong
813	*A. baumannii*	Hong Kong
814	*A. baumannii*	Hong Kong
815	*A. baumannii*	Hong Kong
819	*A. baumannii*	RIE, Edinburgh
ZT 73	*A. haemolyticus*	RIE, Edinburgh
ZT 94	*A. haemolyticus*	SGH, Glasgow
ZT 102	*A. baumannii*	SGH, Glasgow
ZT 106	*A. baumannii*	SGH, Glasgow
ZT 145	*A. junii*	RIE, Edinburgh
ZT 253	*A. junii*	RIE, Edinburgh
ZT 259	*A. baumannii*	RIE, Edinburgh
ZT 264	*A. baumannii*	RIE, Edinburgh
ZT 265	*A. junii*	RIE, Edinburgh

RIE; Royal Infirmary of Edinburgh, SGH; Southern General Hospital, Glasgow

6.3 Antimicrobial susceptibility testing

The MIC range of sulbactam was 0.5 - >16 mg/L. A total of 14 of the 35 isolates (38.9%) had MIC values above the concentration of sulbactam (4 mg/L) that is routinely used in combination with ampicillin (Table 6.2). This total included all imipenem-resistant isolates that produced the ARI-2 β-lactamase. As expected, the lowest MIC values were demonstrated with the isolates from Scotland (MIC range 0.5-2 mg/L, although a slightly higher MIC value was detected with the imipenem-resistant isolate 792. The highest MIC values were observed with the isolates from Argentina (MIC range 4 - >16 mg/L). Tazobactam demonstrated considerable less activity, with all isolates demonstrating higher MIC values (MIC range 2 - >16 mg/L) compared with sulbactam.

Table 6.2 MIC values of sulbactam, tazobactam and imipenem against *Acinetobacter* clinical isolates

	MIC values (mg/L)		
Isolate	**Sulbactam**	**Tazobactam**	**Imipenem**
779	8	>16	8
780	4	>16	8
781	4	>16	8
782	8	>16	8
783	8	>16	16
784	8	>16	16
785	8	>16	32
786	4	>16	8
787	8	16	16
788	8	>16	8
789	16	8	0.25
790	>16	>16	16
791	>16	>16	32
792	4	>16	16
803	2	8	1
804	1	16	0.25
806	16	>16	128
807	1	8	0.25
808	2	16	2
809	8	>16	8

Table 6.2 continued

Isolate	MIC values (mg/L) Sulbactam	Tazobactam	Imipenem
811	4	>16	8
812	2	>16	8
813	2	>16	4
814	2	>16	4
815	2	8	2
819	8	>16	1
ZT 73	1	16	0.06
ZT 94	1	8	0.06
ZT 104	2	8	0.12
ZT 106	1	16	0.06
ZT 145	1	8	0.12
ZT 253	1	16	0.12
ZT 259	0.5	2	0.12
ZT 264	2	16	0.12
ZT 265	2	>16	0.12

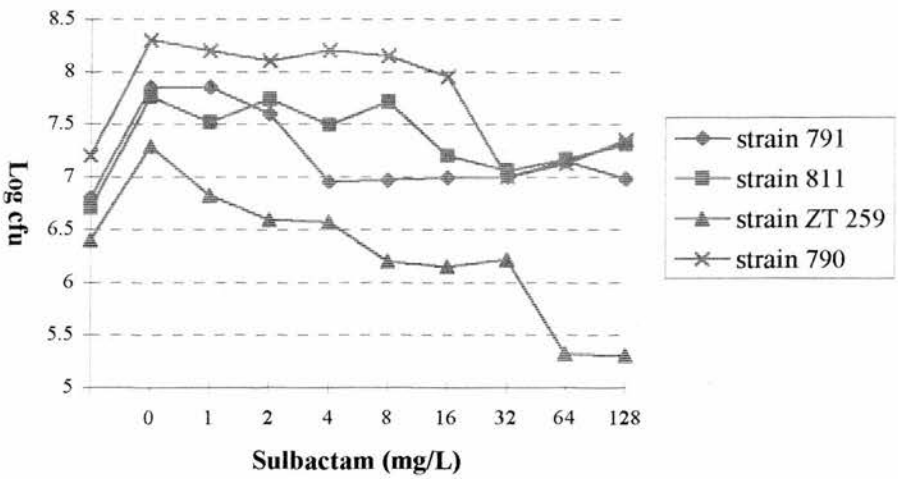
6.4 Investigation of the inhibitory effect of sulbactam on clinical isolates of *A. baumannii*

Three imipenem-resistant isolates demonstrating MIC values of sulbactam of 4 mg/L (isolate 811) and >16mg/L (isolates 790 and 791), and 1 imipenem-sensitive isolate with a MIC value of sulbactam of 0.5 mg/L (ZT 259), were challenged with increasing doses of sulbactam (range 1-128 mg/L) over a 3 hour period.

A minimal inhibitory activity of sulbactam was detected against isolates 790 and 811, although the growth rate of each increased slightly at a concentration of >32 mg/L (Figure 6.1). The activity of sulbactam against isolate 791 was greatest at 4 mg/L (1 log decrease); however, no further decrease in growth rate was observed at higher concentrations. Significant inhibitory activity was demonstrated against isolate ZT 259 at all concentrations of sulbactam (2 log decrease) (Figure 6.1). These findings indicated that for the sulbactam-resistant isolates 790, 791 and 811 (MIC values

of ≥ 4 mg/L), sulbactam did not have a significant bactericidal effect at concentrations above their MIC values. However, the growth rate of the sensitive isolate ZT 259 (MIC value of sulbactam of 0.5 mg/L) continued to decrease at concentrations above its MIC value.

Figure 6.1 Dose response curve of isolates 790, 791, 811 and ZT 259 following challenge with sulbactam



6.5 Investigation of the *in vitro* activity of sulbactam in combination with imipenem against imipenem-resistant clinical isolates of *A. baumannii*

The combined activity of imipenem and sulbactam against isolates 790, 791 and 811, was evaluated by chequerboard titration.

Synergy was detected with a combination of imipenem 8 mg/L and sulbactam 2.0 mg/L (ΣFIC = 0.56) against isolate 790 (Figure 6.2.1). Partial synergy was demonstrated with a combination of imipenem 12 mg/L and sulbactam 1.5 mg/L (ΣFIC = 0.83) against isolates 791 (Figure 6.2.2) and 811 (Figure 6.2.3). Although

this study involved only 3 isolates, these results suggested that the enhanced activity of imipenem in combination with sulbactam may prove to be therapeutically useful against imipenem-resistant isolates.

Figure 6.2.1 *In vitro* activity of combinations of imipenem and sulbactam against isolate 790

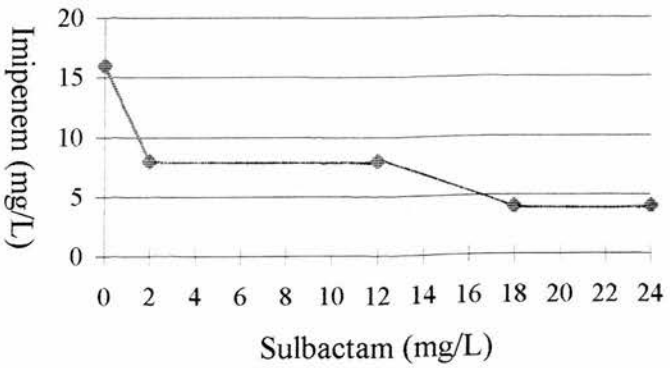


Figure 6.2.2 *In vitro* activity of combinations of imipenem and sulbactam against isolate 791

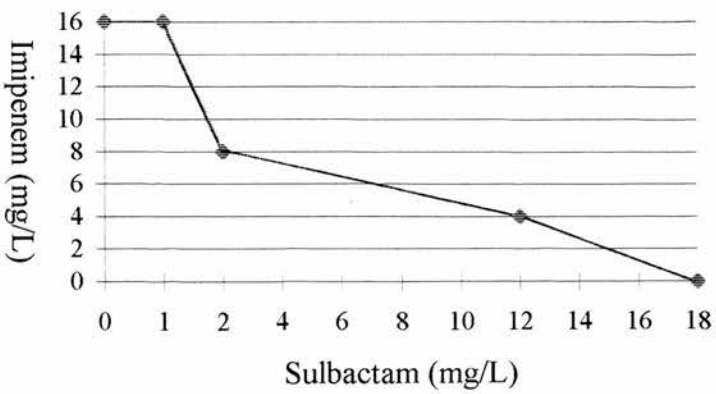
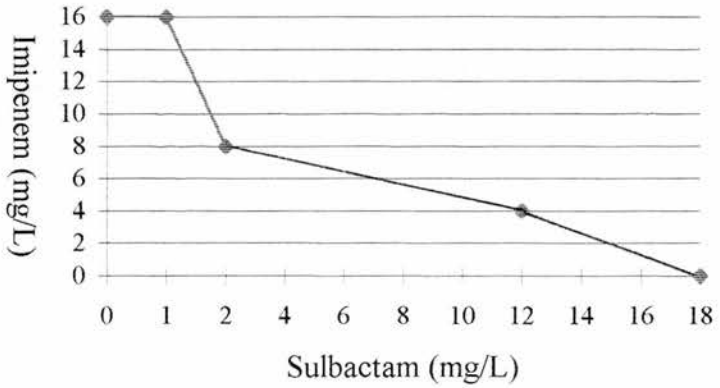


Figure 6.2.3 *In vitro* activity of combinations of imipenem and sulbactam against isolate 811



6.6 Bactericidal *in vitro* activity of imipenem and sulbactam combinations against imipenem-resistant clinical isolates of *A. baumannii*

The activity of imipenem and sulbactam alone at 32 mg/L and 128 mg/L respectively had no effect against isolate 790 (Figure 6.3.1). However, an increased inhibitory effect was observed with imipenem and sulbactam together (1.5 log decrease) after 4 hours, and it can be seen that this combination produced bactericidal activity by 5 hours (3.5 log decrease). This suggests that there is bactericidal synergy with these drugs with this strain.

Against isolate 791, sulbactam alone at 8 mg/L had a bacteriostatic effect after 5 hours (<0.5 log decrease) (Figure 6.3.2). After 4 hours, a bacteriostatic effect was observed with imipenem alone at 12 mg /L. The combined activity of imipenem and sulbactam produced no greater effect on viability than imipenem alone (<0.5 log decrease). This suggests that the two drugs together do not work synergistically to give bactericidal activity.

Figure 6.3.1 Bactericidal activity of imipenem and sulbactam against isolate 790

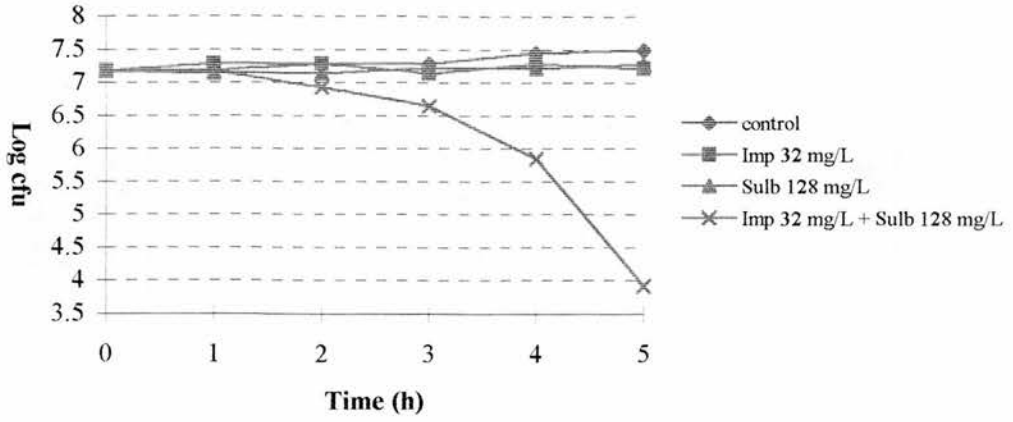
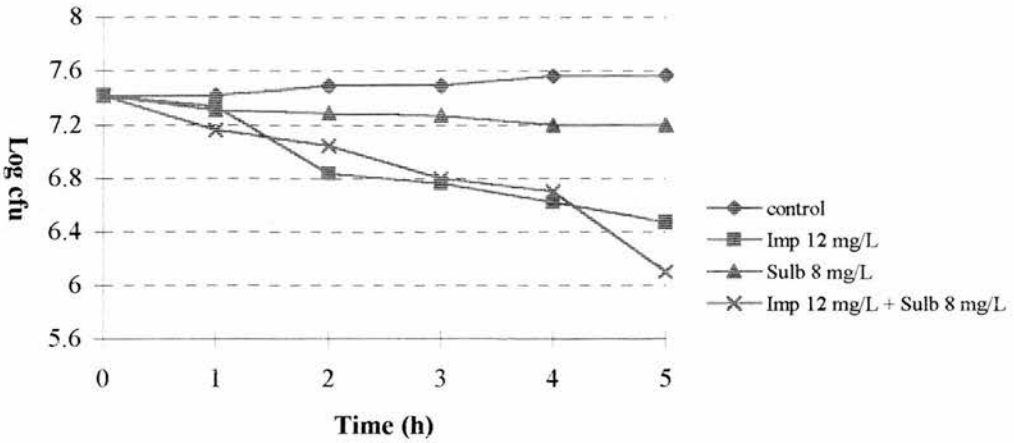


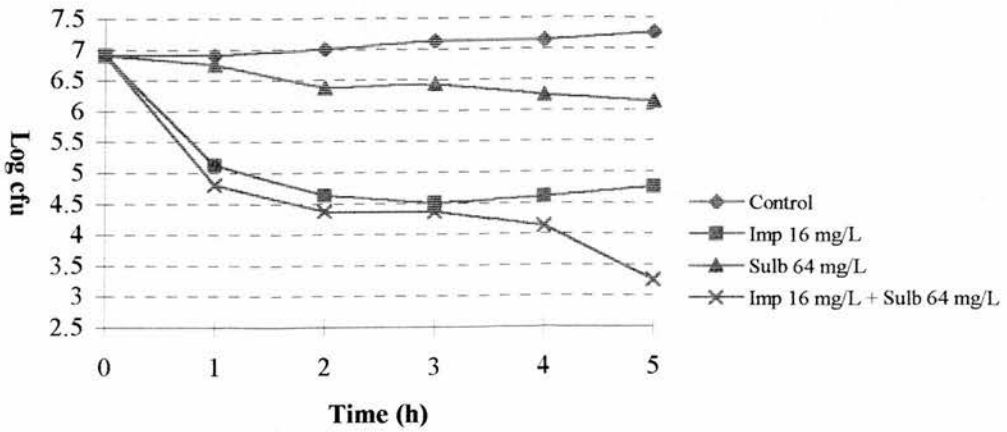
Figure 6.3.2 Bactericidal activity of imipenem and sulbactam against isolate 791



A limited bactericidal effect was obtained with imipenem at 16 mg/L against isolate 811 (2.5 log decrease) over the 5 hour period (Figure 6.3.3). Sulbactam alone at 64 mg/L had only a bacteriostatic effect against this isolate after 5 hours (1 log decrease). The addition of sulbactam did not enhance the bactericidal effect seen with imipenem, as a bacteriostatic response similar to that of sulbactam alone was observed with the

combination of imipenem and sulbactam after 5 hours (1.5 log decrease). This suggests that sulbactam could antagonise the action of imipenem in some strains.

Figure 6.3.3 Bactericidal activity of imipenem and sulbactam against isolate 811



In summary, the combination of imipenem and sulbactam produced the greatest bactericidal effect against isolate 790. The addition of sulbactam did not enhance the low bactericidal activity of imipenem observed against isolate 811, and no bactericidal activity was observed with the imipenem/sulbactam combination against isolate 791.

CHAPTER 7

DISCUSSION

7.1 Bacterial isolates

A total of 54 randomly-collected clinical isolates of *Acinetobacter* spp. from Argentina, Turkey, Hong Kong, Spain and Singapore were investigated in this study. The majority were identified as *A. baumannii*, which is in agreement with the findings of other studies that have reported *A. baumannii* as the most prevalent species found in clinical isolates (Traub, 1989; Jang *et al.*, 1999; Villari *et al.*, 1999). However, it should be noted that the API 20NE identification system used in this thesis does not reliably distinguish the different *Acinetobacter* genomic species, particularly strains belonging to the *A. baumannii-A. calcoaceticus* complex (Bergogne-Bérézin and Towner, 1996).

The majority of isolates (33%) originated from Buenos Aires, Argentina (18 in total), and from Izmir, Turkey (15 in total). In Argentina, multi-resistant *A. baumannii* is now the most important Gram-negative pathogen in the ITU (C. Bantar, personal communication). An antimicrobial surveillance program of 556 strains from hospitalised patients in 6 Latin American countries reported that more than half of Gram-negative isolates were non-fermentative species, of which *A. baumannii* was the most frequently isolated species (Sader *et al.*, 1998). A recent survey of aerobic Gram-negative isolates from ITUs in 8 hospitals in Turkey reported isolation rates of *Acinetobacter* spp. of between 10-23% (Günseren *et al.*, 1999). Indeed, an increase in general in the incidence of *Acinetobacter*-associated infections is well documented. A recent review of ITUs in the UK reported that 45 out of 70 units isolated *Acinetobacter* spp. (Humphreys and Towner, 1997), and in a surveillance study of ITUs in 5 European countries, *Acinetobacter* spp. accounted for 2-10% of Gram-negative isolates; the highest incidence was observed in France (Hanberger *et al.*, 1999). Acinetobacters are now the third most frequently isolated pathogen among hospitalised patients with pneumonia in Latin America (Sader *et al.*, 1998).

7.2 Antimicrobial susceptibility profiles of *Acinetobacter* clinical isolates

Antimicrobial susceptibility studies identified a high level of resistance in all isolates, which were resistant to the majority of antibiotics tested. Resistance rates of 100% were detected for both amoxycillin and amoxiclavulanic acid, and for cephaloridine. The majority were resistant to both cefotaxime and ceftazidime (resistance rates of 96.3% and 98.1% respectively). Both ciprofloxacin and gentamicin demonstrated poor activity against these isolates (resistance rates of 91% and 72% respectively). Multi-resistance is now a common feature among the majority of reported *A. baumannii* strains (Günseren *et al.*, 1999; Sader *et al.*, 1998; Biendo *et al.*, 1999). The excessive use of antimicrobials in the hospital setting has undoubtedly provided a strong selective force that has contributed to the current levels of resistance. Clinical isolates of *Acinetobacter* from community-acquired infections do not appear to possess the same resistance profiles as those of isolates from nosocomial infections (Gerner-Smidt, 1987). In addition, several studies have correlated antibiotic use with a corresponding increase in resistance levels in clinical isolates (Joly-Guillou *et al.*, 1990; Kolar *et al.*, 1999; Villers *et al.*, 1998).

A total of 69% of all isolates demonstrated MIC values of imipenem above the breakpoint value (4 mg/L), of which the highest percentages of resistance were observed in the isolates from Argentina (89%) and Singapore (82%). Although several studies have declared the carbapenems as the only suitable therapy against many multi-resistant isolates of *Acinetobacter* spp. (Mokaddas *et al.*, 1998; Günseren *et al.*, 1999), there are now equivalent numbers of reports that demonstrate an increase in resistance to this class of antibiotic (Sader *et al.*, 1998; C. Bantar, unpublished data). A recent survey of resistance among clinical isolates of *Acinetobacter* in Spain over a 6 year period has highlighted this growing trend. The study reported an overall average increase in antibiotic resistance levels of 42% between 1991 and 1996. Alarmingly, however, the highest increase was detected

with imipenem resistance, which rose from 1.3% in 1991 to 80% in 1996 (Ruiz *et al.*, 1999).

A significantly lower level of resistance was observed to meropenem (38.9%), although 46.3% of all isolates demonstrated MIC values of this carbapenem at the recommended breakpoint value (Working Party on Antibiotic Sensitivity Testing, 1998a), and approximately 38% of all resistant isolates had MIC values of meropenem that were either equal to or greater than those of imipenem.

Although the primary target of both carbapenems in *E. coli* is PBP2, meropenem also has an affinity for PBP3 in *P. aeruginosa* (Kitzis *et al.*, 1989). Meropenem has also been demonstrated to penetrate the outer membrane of Enterobacteriaceae and *Pseudomonas* spp. via more than one type of OMP (Kitzis *et al.*, 1989). This, along with its PBP affinity pattern, may explain the enhanced activity of meropenem against these bacteria.

Although these results correlate with the current view that resistance to meropenem is less common than to imipenem (Livermore, 1997; Jones and Pfaller, 1998), it is alarming that such a large percentage of isolates demonstrated high MIC values of this carbapenem. Indeed, the appearance of resistance to meropenem has been documented. In *P. aeruginosa*, resistance has been attributed to loss of the D2 OMP (Buschner *et al.*,1987). In addition, this antibiotic appears to be a substrate of the MexAB-OprM efflux pump in this species, and it has been demonstrated to select for strains that are over-producers of this pump as a secondary resistance mechanism in OprD-deficient strains (Köhler *et al.*, 1999). Expression of high levels of metallo-enzymes in *S. maltophilia* and *Aeromonas hydrophila* can result in resistance to meropenem (Sanders *et al.*, 1989), as can expression of chromosomal β-lactamase in combination with loss of the D2 porin in *P. aeruginosa* (Livermore, 1992).

Interestingly, one isolate from Spain (806) demonstrated unusually high MIC values of both carbapenems (128 mg/L of both). Resistance to meropenem in *Acinetobacter*

spp. has been reported. Following treatment with this carbapenem, an increase in MIC value from <1 mg/L to >32 mg/L was observed in a clinical isolate of *Acinetobacter* (also isolated in Spain) (Nunez *et al.*, 1998). Sader *et al* (1998) also reported equivalent resistance levels of both carbapenems in clinical isolates of *Acinetobacter*.

7.3 The β-lactamases of *Acinetobacter*

Analysis of the β-lactamase content of imipenem-resistant isolates by IEF revealed the presence of β-lactamase bands of pI >8.0 in 98%. These were presumed to be chromosomally-encoded cephalosporinases because of their high pI. A number of these β-lactamases have been identified in *Acinetobacter* spp. and four have been named ACE-1, 2, 3 and 4, the pI values of which range from 7.3 and 7.7 (ACE-4) to 8.6 and 8.8 (ACE-1) (Hood and Amyes, 1991). Other cephalosporinases of high pI values have also been described (Morohoshi and Sato, 1977; Hikida *et al.*, 1989; Blechschmidt *et al.*, 1992; Perilli *et al.*, 1996). A study by Vila *et al* (1993) identified cephalosporinase activity in 98% of clinical isolates of *A. baumannii* based on IEF bands with a pI value of greater than 8.0. Cephalosporinases were the only enzymes visualised in 2 of the imipenem-resistant isolates from Turkey and 6 from Singapore.

A total of 22 (54%) of the 41 imipenem-resistant isolates also produced β-lactamase bands that co-focused with the class A β-lactamase TEM-1 (pI 5.4). This enzyme was first identified in *Acinetobacter* epidemic strains in the early 1980s, and was responsible for ampicillin resistance observed in these strains (Philippon *et al.*, 1980). The closely related TEM-2 enzyme was identified soon after (Devaud *et al.*, 1982).

The inhibitor overlays of this β-lactamase were in agreement with the properties of TEM-1 in that it was inhibited by the class A inhibitor clavulanic acid, and by BRL 42715, an inhibitor of serine active-site β-lactamases. An additional class A β-lactamase (CARB-5) that hydrolyses both ampicillin and carbenicillin has been identified in some *Acinetobacter* strains (Joly-Guillou *et al.*, 1988). This enzyme has

a pI value of 6.3, however no bands comparable to that of CARB-5 were detected in any of the isolates.

In the majority of isolates from Singapore and 2 isolates from Turkey, a β-lactamase of pI >8.0 that resembled a chromosomal cephalosporinase was the only enzyme observed, although 3 isolates from Singapore also produced a TEM-1 β-lactamase. The MIC range of imipenem observed in these isolates was 4.0-16 mg/L. Although isoelectric focusing had detected partial inhibition of the cephalosporinase bands following an overlay of imipenem, the microbiological assays provided no evidence that these β-lactamases alone or in combination were involved in imipenem-resistance observed in these strains therefore, another mechanism other than β-lactamase activity was assumed to be responsible.

Alterations in the outer membrane permeability have been well documented as a cause of resistance in Gram-negatives, and it remains the most common mechanism of resistance to imipenem. In *P. aeruginosa*, for example, loss of expression of the OprD porin results in resistance to imipenem (Lynch *et al.*, 1987). A decrease in the expression of an outer membrane protein has been attributed to imipenem resistance in 2 clinical isolates of *A. baumannii* (Clark, 1996), and a major 40 kDa outer membrane protein (OmpAb) has recently been characterised and appears to play an important role in the diffusion properties of the outer membrane of *A. baumannii* (Jyothisri *et al.*, 1999). Whether the loss of, or a decrease in, the production of this protein can result in antibiotic resistance remains to be determined. The presence of a small number of small-sized porins has been suggested as one of the causes of the resistance profile of *Acinetobacter* spp. (Sato and Nakae, 1991). This study also demonstrated that diffusion rates of the carbapenems through the outer membranes of *Acinetobacter* were less than 2% of that detected in *E. coli*.

It is known that multicomponent efflux systems act synergistically with low permeability to produce the intrinsic resistance observed in *P. aeruginosa* (Nikaido, 1996), and the carbapenems are included in the substrate range of one such system

(MexEF-OprN) (Masuda *et al.*, 1995). Whether a similar system exists in *Acinetobacter* spp. has yet to be determined.

7.3.1 Discovery of unknown β-lactamase bands in imipenem-resistant isolates

Two β-lactamase bands of pI values 8.65 and approximately 8.9 were visualised in isolate 806 from Spain. These bands were very similar in pI to the bands of ACE-1 (pI values of 8.6 and 8.8) (Hood and Amyes, 1991). Isolate 806 was unusual in that it demonstrated very high MIC values to both imipenem and meropenem (128 mg/L of both). Interestingly, IEF inhibitor overlays of imipenem did not diminish the activity of the band of pI 8.65, but the band of pI 8.9 was completely inhibited, which suggested that imipenem binding had occurred. This raised the possibility that these bands comprised two separate β-lactamases, and that the band of pI 8.9 was involved in carbapenem resistance, possibly in combination with other mechanisms. A β-lactamase of pI 8.0 has recently been implicated in carbapenemase resistance in a clinical isolate of *Acinetobacter* spp. from Spain (Afzal-Shah *et al.*, 1999a).

Three isolates from Hong Kong (812, 813 and 814) produced an identical band of pI 8.25 with associated satellite banding that resembled ACE-3 (pI 8.1 and 8.2). However, the β-lactamase bands detected in these isolates were inhibited by an IEF overlay of imipenem. Isolate 876 from Turkey produced a β-lactamase of pI 7.8 that was also inhibited by an imipenem overlay. This suggested that β-lactamase activity could be involved in carbapenem resistance in these isolates.

High-level expression of a chromosomally-encoded class C β-lactamase combined with reduced outer membrane permeability has been associated with carbapenem resistance among members of Enterobacteriaceae (Lee *et al.*, 1991; Marinardi *et al.*, 1997; Raimondi *et al.*, 1991). The possibility existed, therefore, that cephalosporinase activity may contribute to imipenem resistance in those isolates in which these were the only enzymes detected. There is no documented evidence that

any of the ACE cephalosporinases play a role in imipenem resistance, although the production of a cephalosporinase in combination with either modification of OMPs or PBPs has been suggested as a cause of imipenem resistance in *A. baumannii* clinical isolates however, these workers did not fully characterise the β-lactamases involved (Dib *et al.*, 1994). To determine whether β-lactamase activity was indeed involved in carbapenem resistance in the isolates from Hong Kong, Turkey and Spain would require further characterisation of their β-lactamases.

7.4 ARI-2 - a novel β-lactamase involved in carbapenem resistance

A β-lactamase of main band pI 7.0 was identified in 61% of imipenem-resistant isolates, which included all of the isolates from Argentina, 6 from Turkey, and one each from Spain and Hong Kong. A β-lactamase of pI 7.0 has also been described in several clinical isolates of *A. baumannii*, however these strains remained susceptible to imipenem with MIC values of ≤1 mg/L (Vila *et al.*, 1993).

The modified microbiological assay using crude β-lactamase extracts of isolates demonstrated inactivation of imipenem, and to a lesser extent meropenem, suggesting that β-lactamase activity was involved in carbapenem resistance observed in these isolates. This assay has also been used to demonstrate inactivation of imipenem by the carbapenemase ARI-1 in a clinical isolate of *A. baumannii* (Paton *et al.*, 1993). Crude β-lactamase extracts from both a TEM-1 producer and an imipenem-sensitive clinical isolate of *A. baumannii* (789) failed to inactivate imipenem and meropenem by this method.

7.4.1 Initial characterisation of ARI-2

An imipenem overlay of IEF gels of crude β-lactamase extracts of strains producing the enzyme of pI 7.0 resulted in loss of activity bands after nitrocephin staining, indicating that imipenem binding had occurred. Further overlays with inhibitors were subsequently employed to determine whether this enzyme belonged to Bush group 2f,

which includes the Ambler molecular class A serine active-site carbapenemases, or Bush group 3, the molecular class B metallo-enzymes (Bush *et al.*, 1995).

β-lactamase activity was not affected by EDTA, a metal ion chelator that inhibits class B β-lactamases, but was affected by BRL 42715, an inhibitor of serine active-site enzymes. These results indicated that the β-lactamase of pI 7.0 was not a metallo-β-lactamase, but instead possessed a serine residue at its active-site. These findings were not surprising since the presence of metallo-enzymes in *Acinetobacter* spp. is extremely rare. There have been isolated reports of metallo-enzymes in clinical isolates of *A. baumannii* from Portugal (Da Silva and Peixe, 1999) and from Cuba (Pérez *et al.*, 1996). Both β-lactamases were characterised based on their hydrolysis of imipenem and inhibition by EDTA; however, the genes that encode these enzymes have yet to be sequenced. A third report from Italy has identified an IMP-1-like enzyme in a single *A. baumanniii* isolate based on hybridisation of chromosomal DNA to a bla_{IMP}-specific probe (Cornaglia *et al.*, 1999), and more recently, a metallo-enzyme has been identified in a clinical isolate of *Acinetobacter* spp. from Hong Kong that does not appear to be related to either IMP-1 or VIM-1 (Afzal-Shah *et al.*, 1999a).

Partial inhibition of the pI 7.0 β-lactamase occurred with clavulanic acid at 1 mM. This inhibitor primarily inhibits class A but not class C β-lactamases (Bush *et al.*, 1995). It also weakly inhibits the activities of OXA-type (oxacillin-hydrolysing) enzymes (Naas and Nordman, 1999), therefore the possibility existed that this β-lactamase belonged to either class A or class D. Several class A β-lactamases exist that are able to hydrolyse imipenem (Livermore, 1997); however, until recently, the carbapenems have not been included in the substrate range of class D enzymes. The inhibitor profile of this β-lactamase was very similar to that of the ARI-1 carbapenemase (pI 6.65), which was originally thought to be a class A enzyme, but has recently been identified as a class D oxacillinase (Donald *et al.*, 2000). The enzyme in this thesis with a pI of 7.0 was subsequently named ARI-2.

7.4.2 Spectrophotometric analysis of induced and non-induced ARI-2-producing strain 790

Spectrophotometrical analysis of crude β-lactamase extracts from a representative strain (790) that produced ARI-2, failed to detect direct imipenem hydrolysis. However, this did not rule out its involvement in imipenem resistance, since this anomaly has also been reported with ARI-1 (Paton *et al.*, 1993). Indeed, analysis of crude extract from the ARI-1-producing strain 6B 92 in the present study confirmed these findings. Following pre-incubation with imipenem, the rate of nitrocephin hydrolysis of crude extract of 790 decreased from 8.39 to 0.32 nmoles hydrolysed min^{-1} mg^{-1} of protein. This suggested that, although hydrolysis of imipenem could not be measured spectrophotometrically, β-lactamase binding of this carbapenem has occurred, resulting in a loss of enzyme activity and a subsequent decrease in the rate of nitrocephin hydrolysis. A similar finding was also detected with ARI-1 (62.43 to 0.33 nmoles hydrolysed min^{-1} mg^{-1} of protein).

The possibility existed that the ARI-2 β-lactamase hydrolysed imipenem at a rate too slow to be detected spectrophotometrically during the 5 minute assay time therefore, the microbiological assay was modified to measure hydrolysis over a period of 90 minutes. The specific activities of crude β-lactamase extracts from strains 790 and 6B 92 were 0.055 and 0.043 nmoles hydrolysed min^{-1} mg^{-1} of protein respectively, which indicated that indeed, both ARI-1 and ARI-2 hydrolysed imipenem slowly at comparable rates.

Induction studies with imipenem at ¼ the strain MIC value did not result in an increase in MIC value of imipenem for either strain 790 or 6B 92. In addition, there were no significant corresponding increases in nitrocephin hydrolysis detected after induction, which suggested that both β-lactamases were non-inducible. β-lactams are known to act as inducers of class C β-lactamases in Gram-negative bacteria, resulting in derepression of β-lactamase and high level resistance to many β-lactams (Stapleton *et al.*, 1995; Bennett and Chopra, 1993). Although inducible β-lactamase genes of

Gram-negative bacteria were considered to be exclusively chromosomal, plasmid-mediated AmpC type β-lactamases have now been found (Bauernfeind *et al.*, 1996; Payne *et al.*, 1992; Tzouvelekis *et al.*, 1993), some of which are inducible (Barnaud *et al.*,1998) and are involved in imipenem-resistance in combination with impermeability (Bradford *et al.*, 1997; Stapleton *et al.*, 1999). Consequently, although the induction studies with strain 790 suggested that ARI-2 was not a class C β-lactamase, they did not provide any evidence as to the genetic location of the gene encoding this enzyme.

7.4.3 Elimination of plasmid-determined resistance by treatment with ethidium bromide

The location of the genetic determinant of ARI-2 was investigated by treating strain 790 with ethidium bromide. This is a trypanocidal drug that preferentially inhibits plasmid synthesis by intercalating between the base pairs of the double helix causing lengthening and unwinding. The use of this agent has been used to eliminate plasmid-encoded antibiotic resistance in staphylococci and enterobacteria (Bouanchaud *et al.*, 1969). In addition, Paton *et al* (1993) demonstrated the loss of imipenem resistance in strain 6B 92 following treatment by ethidium bromide.

A cure rate of 36.8% was obtained with strain 790, and 41% with the ARI-1-producing strain 6B 92 which was included as a positive control. These rates were much higher than previous reports employing this method with *Acinetobacter*. Goldstein *et al* (1983) detected a cure rate of only 0.3% with *A. calcoaceticus*. Paton *et al* (1993) obtained a cure rate of 10% of imipenem resistance with 6B 92 compared with 41% in this study. Indeed, these workers suggested that resistance plasmids in *Acinetobacter* spp. may not be as easily eliminated by this method of curing as those present in Enterobacteriaceae (Paton, 1994); however, the cure rates of nearly 37% and 41% obtained in this study are comparable with those obtained with *E. coli* (Bouanchaud *et al.*, 1969).

The MIC values of cured colonies of strain 790 provided additional evidence that carbapenem resistance was indeed associated with the loss of a plasmid. The MIC value of imipenem decreased to a susceptible level (16 mg/L to 0.5 mg/L). A less significant decrease however, was observed with meropenem (4 mg/L to 1.0 mg/L). None of the other β-lactams tested were affected by curing as indicated by comparable MIC values for both cured and parent colonies. The decrease in carbapenem MIC values were in agreement with Paton and colleagues (1993) who also obtained similar results with 6B 92; however, in contrast to the findings of these workers, who also noticed a decrease in MIC values of penicillins after curing, no corresponding decrease in MIC values of this class was observed with strain 790. This suggested that although similarities existed with both ARI-1 and ARI-2, they were not identical β-lactamases.

Isoelectric focusing demonstrated loss of the ARI-2 β-lactamase in cured colonies of strain 790. The β-lactamase band of pI >8.0 (chromosomal cephalosporinase) was present in both cured and parent extracts, which suggested that it was not involved in imipenem resistance, but was responsible for the cephalosporin resistance observed in this strain. These findings are in agreement with those observed with 6B 92, which also retained its chromosomal cephalosporinase and resistance to the cephalosporins following ethidium bromide curing (Paton *et al.*, 1993).

Interestingly, the TEM-1 β-lactamase (pI 5.4) was also retained in the cured colonies. This enzyme is plasmid-encoded (Bush, 1989b), and subsequently would be expected to be lost after curing. However, colonies were tested for the loss of imipenem resistance, and not penicillins. Therefore, the possibility existed that in the cured colonies tested, ethidium bromide curing had not eliminated the plasmid encoding this particular enzyme.

When crude β-lactamase extracts of cured colonies of strain 790 were subjected to the microbiological assay, no inactivation of imipenem was observed. In addition, the modified microbiological assay detected an 11-fold decrease in the rate of imipenem

hydrolysis compared with the parent strain. Together, these findings provided strong evidence that the ARI-2 β-lactamase was indeed the major mechanism of imipenem resistance and that the gene encoding this enzyme was encoded by a plasmid. However, the possibility still existed that a change in outer membrane permeability or alterations in PBPs could be involved in imipenem resistance. To investigate this hypothesis, a series of susceptibility tests were employed with the serine active-site inhibitor BRL 42715. By determining the MIC values of both carbapenems in the presence of this agent, the role of ARI-2 activity in carbapenem resistance could be evaluated. The results demonstrated that β-lactamase inhibition by BRL 42715 resulted in a MIC value of imipenem comparable to that observed with the cured strain, suggesting that without the activity of ARI-2, the strain becomes susceptible to this carbapenem. It is interesting to note that there was not as significant a decrease in the MIC value of meropenem in the presence of this inhibitor (from 8 mg/L to 4 mg/L), which suggests that, in this particular strain, β-lactamase activity is not the sole mechanism of reduced susceptibility to this particular carbapenem. Paton *et al* (1993) confirmed that activity of the ARI-1 β-lactamase was responsible for imipenem resistance in strain 6B 92 by this method however, in contrast to ARI-2, these workers also demonstrated that this β-lactamase was also responsible for the high MIC value of meropenem observed in this strain.

In order to obtain additional evidence that the genetic determinant of ARI-2 was indeed plasmid-located, a series of experiments were employed in an attempt to transfer the resistance plasmid. Although the presence of a plasmid of approximately 40 kb in size was visualised in the parent strain, but not in cured colonies of strain 790, all efforts to transfer this plasmid at both 25°C and 37°C were unsuccessful. In addition, transformation experiments with the highly competent *Acinetobacter* sp. BD413-2 strain also failed.

Transfer of the plasmid harbouring the ARI-1 gene has been carried out successfully using these methods to strains C4161 and BD413, and exclusively at 25°C, which suggests that the plasmid encodes a temperature-sensitive transfer mechanism (Scaife

et al., 1995). The inability to transfer the plasmid observed in strain 790 may reflect a lack of conjugative functions associated with it, and it is possible that an additional mobilising plasmid is needed (Chopade *et al.*, 1985). Alternatively, the gene encoding the ARI-2 enzyme may be located on a mobile genetic element that was subsequently lost from the plasmid. It is known that chromosomally-located transposons carrying multiple antibiotic resistance genes are present in *Acinetobacter* spp. (Towner, 1991), and plasmid-encoded resistance genes, including the genes encoding the metallo-enzymes IMP-1 (Arakawa *et al.*, 1995) and VIM-1 (Lauretti *et al.*, 1999) have been found on integron structures.

The failure to transform *Acinetobacter* sp. BD413-2 suggests that perhaps the plasmid observed in strain 790 was larger than initially estimated, and therefore proved too large to be transferred efficiently. Plasmid transfer was not attempted with recipients other than *Acinetobacter*. Both Paton *et al* (1993) and Scaife *et al* (1995) failed to transfer the ARI-1 plasmid to *E. coli* or *P. aeruginosa* strains, which is in agreement with others that have reported an inability of *Acinetobacter* spp. to transfer indigenous plasmids to Enterobacteriaceae (Towner, 1991).

The inability of these experiments to demonstrate the transfer of imipenem-resistance did not invalidate the theory that the ARI-2 gene was located on a plasmid. Rather, they only confirmed the findings of other workers, that conjugation studies in *Acinetobacter* spp. are notoriously difficult (Goldstein *et al.*, 1983; Towner, 1991).

7.4.4 Partial purification and estimation of the M_r of ARI-2

Attempts to separate the ARI-2 β-lactamase from the other enzymes present in strain 790 were only partially successful in that it could be separated from the cephalosporinase but not from the TEM-1 β-lactamase. This suggested that ARI-2 was similar in M_r to this enzyme. This was confirmed by SDS-PAGE with subsequent renaturation of enzyme activity, which demonstrated 2 bands of activity in close proximity to each other, with a third band above them.

The M_r of the lower band (29.8 kDa) corresponded to that of the TEM-1 β-lactamase (Bush *et al.*, 1995). The band near the top of the gel was approximately 53 kDa, which is within the size range reported for the chromosomal cephalosporinases of *Acinetobacter* spp., and most closely resembles the cephalosporinase of 58 kDa described in strain 6B 92 (Paton *et al.*, 1993). Cephalosporinases with M_r values of 30,000 and 38,000 have been described (Morohoshi and Saito, 1977; Hikida *et al.*, 1989), and the ACE β-lactamases described by Hood and Amyes (1991) range from 35,000 (ACE-3) to >500,000 (ACE-1 and ACE-4) in size.

The third band, presumed to be that of ARI-2 had an M_r value of 35.5 kDa, which is a higher value than that reported for the ARI-1 β-lactamase (23 kDa) (Paton *et al.*, 1993), although the value for ARI-1 was estimated by gel filtration rather than SDS-PAGE, a method that can underestimate the molecular weights of β-lactamases (Bush, 1989a). Among the serine active-site carbapenemases that have been characterised, the ARI-2 β-lactamase was more similar in molecular mass to the class A IMI-1 enzyme (M_r of 32 kDa by SDS-PAGE), which also shares a similar pI value (7.05) to that of ARI-2 (Rasmussen *et al.*, 1996). Indeed, all of the serine active-site carbapenemases, including those belonging to Bush group 2d, share similar molecular masses in the range 28-32 kDa (Bush *et al.*, 1995; Hornstein *et al.*, 1997; Afzal-Shah *et al.*, 1999b).

7.4.5 Biochemical characterisation of ARI-2

Despite several efforts employing different separation conditions, further purification of the ARI-2 β-lactamase was unsuccessful however, this problem was solved by employing another strain (788) from Argentina that contained both a cephalosporinase and ARI-2, but lacked the TEM-1 β-lactamase.

The substrate profile of ARI-2 demonstrated that this β-lactamase was predominantly a penicillinase. The highest V_{max} values were observed for ampicillin and oxacillin, although the V_{max}/K_m ratio for the latter was over 2-fold lower than that observed for

cephaloridine. Interestingly, slow hydrolysis of imipenem (but not meropenem) was detected with the purified enzyme. Indeed, the lowest K_m value (demonstrating a high affinity) was detected with this carbapenem (11 μM). The V_{max} value for imipenem (46.8 nmoles hydrolysed min^{-1} ml^{-1}) was 1.5-fold lower than that detected for ARI-1 (Donald *et al.*, 1999), but almost 2-fold higher than that reported for a carbapenemase from a clinical isolate of *A. baumannii* from Buenos Aires (Afzal-Shah *et al.*, 1999b). Incidentally, failure to detect meropenem hydrolysis by spectrophotometric means has also been documented with both these β-lactamases (Donald *et al.*, 1999; Afzal-Shah *et al.*, 1999b).

The inability to detect imipenem hydrolysis spectrophotometrically with crude β-lactamase extract from ARI-2-producing strains may have been as a result of the enzyme being too dilute. Considerable enzyme dilution is known to occur during the extraction procedure (Livermore, 1985). It had been noted that in preparing β-lactamase extracts of both strains 788 and 790, the cells resisted sonication therefore, the possibility existed that an insufficient β-lactamase yield may have contributed to this anomaly. The cell walls of *Acinetobacter* spp. are notoriously difficult to break open (Towner, 1996). Interestingly, the direct hydrolysis of imipenem by ARI-1 has recently been reported (Donald *et al.*, 1999), even though initial studies by Paton *et al* (1993) failed to demonstrate this. The former workers achieved this only after purification and subsequent concentration of the enzyme.

Slow hydrolysis of both oxacillin and cloxacillin was demonstrated, which suggested that the ARI-2 β-lactamase was not a Bush group 2f carbapenemase, since none of the enzymes in this group have been shown to hydrolyse either antibiotic, but that it may be related to one of the OXA-type enzymes of Bush group 2d, although such enzymes tend to hydrolyse these β-lactams far more effectively (>50% that for benzylpenicillin) (Bush *et al.*, 1995) than was demonstrated with ARI-2. However, there are exceptions to this criterion, notably OXA-4 (Philippon *et al.*, 1983), and the AmpS oxacillinase from *Aeromonas jandei* that hydrolyses cloxacillin poorly (Walsh *et al.*, 1995). Another characteristic of oxacillinases is that they are not as well

inhibited by clavulanic acid as class A β-lactamases (Bush *et al.*, 1995), a feature that ARI-2 also demonstrated. Indeed, an identical ID_{50} value (100 μM) was also detected for ARI-1 by Paton *et al* (1993) using this inhibitor, compared with a value of 0.5 μM for the class A β-lactamase TEM-1.

Further evidence to suggest that the ARI-2 β-lactamase was OXA-derived was provided by the observation that inhibition of activity occurred with sodium chloride (ID_{50} value of 3.2 mM), a characteristic shared by oxacillinases (Naas and Nordmann, 1999). Cloxacillin (an inhibitor of class C β-lactamases) was not an effective inhibitor of enzyme activity, as demonstrated by a ID_{50} value that was almost 2000-fold higher than that observed for the ACE cephalosporinases (Hood and Amyes, 1991). Low ID_{50} values were detected for imipenem and meropenem, indicating high enzyme affinity for both carbapenems.

The majority of OXA-type β-lactamases are found on plasmids and are subsequently found in a large number of different species (Naas and Nordman, 1999). However, the presence of these enzymes in *Acinetobacter* spp. has only been a recent event. In addition, an alarming characteristic of the majority of the enzymes discovered so far in this genus, is an ability to confer resistance to imipenem (Hornstein *et al.*, 1997; Afzal-Shah *et al.*, 1999a; Afzal-Shah *et al.*, 1999b; Donald *et al.*, 2000), although the first sequenced oxacillinase to be reported in *Acinetobacter* spp. (OXA-21) was from an imipenem-sensitive strain (Vila *et al.*, 1997a). The ARI-1 β-lactamase was originally classified as an Ambler class A carbapenemase (Paton *et al.*, 1993); however, this too has now been demonstrated to be an OXA-type enzyme (Donald *et al.*, 2000).

The substrate profile of ARI-2 was similar to that of the other oxacillinases described in *Acinetobacter* in that it was not a hydrolyser of the second and third-generation cephalosporins. The OXA-type β-lactamases of group 2d differ in their ability to confer resistance to the extended-spectrum cephalosporins. Derivatives of OXA-10 (OXA 11, -14, -16 and -17), and OXA-15 (a derivative of OXA-2), have broader

activity than the other oxacillinases (Naas and Nordman, 1999). In contrast, ARI-2 appears similar in substrate range to ARI-1 (Donald *et al.*, 2000), and to other narrow-spectrum oxacillinases (Naas and Nordmann, 1999).

7.4.6 Molecular characterisation of ARI-2

7.4.6.1 Investigation of homology with other serine active-site β-lactamases

The bands of ARI-2 as observed by IEF were identical to those of the extended-spectrum SHV-3 β-lactamase. Therefore, a series of PCR reactions and hybridisation experiments were performed to rule out the possibility that it was an SHV-derived enzyme. The results of these indicated it was not SHV-derived, and the identical banding pattern was attributed to the fact that many β-lactamases share identical pI values even though they are not related to each other (Bush *et al.*, 1995). The biochemical analysis of the ARI-2 β-lactamase suggested that it belonged to Ambler class D. OXA-21 was identified in an imipenem-sensitive *Acinetobacter* clinical isolate, and shares an identical pI value to that of ARI-1 (Vila *et al.*, 1997a), therefore it was theoretically possible that ARI-2 was a mutated form of this β-lactamase that had acquired the ability to confer resistance to imipenem. However, this hypothesis was unfounded since PCR with intragenic primers, designed from the gene of OXA-21, failed to detect any product with ARI-2-producing strains. OXA-21 is included in a phylogenetic group that includes OXA-2, OXA-3, OXA-15 and OXA-21 (Sanschagrin *et al.*, 1995; Naas and Nordman, 1999), and shares 99.6% amino acid homology with the OXA-3 β-lactamase (Vila *et al.*, 1997a).

Attention was subsequently directed towards identifying possible homology with the gene encoding ARI-1. However, PCR analysis with intragenic primers again failed to detect a product, even when reduced annealing temperatures were employed to encourage the primers to anneal to sequences that have less similarity. This was surprising since non-specific products were expected, at least with strain 6B 92 that was present as a positive control. However, the possibility still existed that the ARI-2

gene may share significant homology with the complete ARI-1 gene sequence therefore DNA:DNA hybridisation studies were employed to investigate this further.

Although initial results were extremely promising in that plasmid DNA from strain 790 demonstrated moderate cross-hybridisation with an ARI-1-specific gene probe, subsequent plasmid DNA preparations failed to produce a signal and, in addition, several chromosomal DNA preparations from the same strain were demonstrated to hybridise with this probe. These findings suggested that perhaps chromosomal contamination had produced the initial result. However, the curing experiments had successfully demonstrated the loss of ARI-2 as a result of plasmid elimination, and a plasmid of approximately 40 kb had been visualised in the parent strain that was not apparent in the cured.

Further hybridisation studies with a degenerate probe for a conserved sequence of bla_{OXA} genes also produced cross-hybridisation with chromosomal DNA but not with plasmid DNA. Consequently, this raised the possibility that the ARI-2 gene was on a mobile element that allowed its transfer between the chromosome and plasmid.

Interestingly, the chromosomal DNA of the cured strain also hybridised, albeit weakly with this probe. One possible explanation could be the presence of a silent copy of the ARI-2 gene on the chromosome that would be detected by hybridisation with this probe. Indeed, silent copies of the carbapenem-resistance gene *cfi*A are known to be carried by a small number of isolates of *Bacteroides fragilis*, in which insertion of a small insertion sequence immediately upstream of the gene results in its activation (Podglajen *et al.*, 1992).

A substantial proportion of antibiotic resistance genes found in Gram-negative bacteria are located within gene cassettes that are usually within integrons (Hall and Collis, 1998). Indeed, a common feature of the majority of oxacillinase genes is that they are located on the variable region of integrons (Philippon *et al.*, 1997; Naas *et al.*, 1998; Naas and Nordmann, 1999). Of the oxacillinases described in

Acinetobacter spp., OXA-21 remains the only one so far to be found associated with an integron (Vila *et al.*, 1997a), although molecular characterisation of many of the other enzymes is ongoing.

PCR amplification with 2 sets of primers designed to anneal to the 5' and 3' ends of the *IntI 1* gene, and to the variable region of class 1 integrons, failed to produce products with strain 790, which suggested that there were no class 1 integrons present in this strain. However, several class 1 integrons do not possess a *qacEΔ1* resistance gene (encoding antiseptic and disinfectant resistance) (Hall and Collis, 1998), to which the Cass 2 primer anneals, therefore no product will be obtained if such integrons are present. Furthermore, the sequence of the region containing the integrase gene and the *attI* recombination site is distinct in each of the three classes of integrons (Recchia and Hall, 1995) thus, primers designed to detect this region in class 1 integrons will not detect those belonging to class 2 or class 3.

Class 1 integrons (of the *sul1* type) are most prevalent in clinical isolates, although the bla_{IMP} gene cassette has been found in both class 1 and class 3 integrons (Arakawa *et al.*, 1995). Subsequently, PCR analysis of ARI-2-producing strains with primers specific for these integrons, or indeed for class 1 integrons that do not contain a *qacE* gene may yet identify an integron location of the ARI-2 gene.

Although initial molecular studies had failed to obtain a handle on the sequence of the ARI-2 gene, they strongly suggested that ARI-2 was indeed a class D enzyme, and, although not identical to ARI-1, some homology existed between the two β-lactamases.

7.4.6.2 N-terminal amino acid sequencing of the ARI-2 protein

A different approach was employed in an attempt to gain an initial sequence region of the gene. Although both sequenced protein bands, from column fractions containing the ARI-2 β-lactamase, failed to demonstrate homology with any β-lactamases, band

A was removed from the investigation since it shared 95% amino acid homology with a ribosomal binding protein. This left the second band (B), and, although no homology was identified with any relevant proteins, it was possible that this sequence represented a signal peptide of the ARI-2 β-lactamase, much in the same way as the N-terminal sequence of ARI-1 proved to be the signal peptide of this enzyme (H. Donald, personal communication). Hybridisation studies with a degenerate probe designed from this sequence however, failed to detect a signal with either ARI-2-producing strains or with the ARI-1 transconjugant strain, even under low stringency conditions, and may have been as a result of the degenerate nature of the probe.

Sequencing of the ARI-1 gene (which has 36% identity with OXA-5 and OXA-10) has revealed an unusual substitution in one of the conserved regions found in OXA-type β-lactamases called the Y-G-N motif, in which tyrosine (Y) has been replaced by phenylalanine (F) (Donald *et al.*, 2000). Indeed, the only other class D carbapenemase (pI value of 9.0) from *Acinetobacter* that has been sequenced, shows a similar identity (40%) with OXA-10, and also has this unique substitution (Bou and Martínez-Beltrán, 1999), which suggests that it may be important in the ability of these enzymes to hydrolyse imipenem. In addition, the Y-G-N motif of the OXA-21 gene (from an imipenem-sensitive *A. baumannii* isolate) is conserved (Vila *et al.*, 1997a). It would therefore be interesting to determine whether this substitution was present in the ARI-2 gene.

7.5 The presence of ARI-2 in clinical isolates from Turkey, Spain and Hong Kong

The presence of β-lactamases identical to ARI-2 in 8 isolates from these countries suggests that this enzyme possesses the potential to transfer horizontally. Indeed, analysis of RFLP patterns of isolates producing ARI-2 indicated that this had occurred with the strains from Buenos Aires. These findings were further supported by the fact that none of the patients from which ARI-2-producing strains had been

isolated had attended any of the other hospital centres, which were geographically remote from each other (J. Smayevsky, personal communication). However, the presence of isolates from clusters C and E in more than one hospital in Buenos Aires, provided evidence that clonal spread had also occurred and indicated that there had been some contact between the hospitals.

Isolate 787 had been identified as *A. junii* by the API 20NE system however, the RFLP pattern of this isolate shared 97% similarity with the *A. baumannii* isolate 790, suggesting that the biotyping of this strain was incorrect.

The presence of this β-lactamase in isolates from several countries is of major concern. Although discovery of the ARI-1 β-lactamase generated interest in the scientific field, it has only been found in a single clinical isolate (Paton *et al.*, 1993), although recent research that has identified ARI-1 as a class D enzyme has renewed interest in this β-lactamase (Donald *et al.*, 2000). The imipenem-hydrolysing oxacillinase reported in France was also from a single isolate (Hornstein *et al.*, 1997). However, more recently, there have been incidences of similar enzymes from multiple isolates world-wide (Afzal-Shah *et al.*, 1999a), although to my knowledge, none of these strains have been analysed to determine whether they are genetically unrelated, and none of the β-lactamases have been found in more than one country. Consequently, the ARI-2 β-lactamase is unique in that it appears to be the first of these enzymes to be associated with world-wide carbapenem resistance.

7.6 Quinolone resistance in *Acinetobacter* spp.

The emergence of *Acinetobacter* species, in particular, *A. baumannii* as important nosocomial pathogens has resulted in an increased reliance on the carbapenems for the treatment of serious infections caused by these organisms. Unfortunately, the recent development of resistance to this class of β-lactams and, in particular, (as the work of this thesis has demonstrated) the emergence of imipenem-hydrolysing β-lactamases has undermined their therapeutic success, with the result that the range of available

antimicrobial agents that are still active against *Acinetobacter* is becoming severely limited. So what are the alternatives? The fluoroquinolones, for example ciprofloxacin, have until recently proven to be highly active against this genus. In 1995, approximately 80% of strains were susceptible to ciprofloxacin, pefloxacin and ofloxacin (Bergogne-Bérézin, 1996); however, resistance has increased within the last decade. Recent research into this class of antibiotics has resulted in a plethora of new fluoroquinolones that are about to emerge into the therapeutic spotlight. The aim of this particular study was therefore to determine the efficacy of some of the newer agents against multi-resistant *Acinetobacter* spp., and to ascertain whether they represent a therapeutic alternative against imipenem-resistant clinical isolates.

Antimicrobial susceptibility testing revealed significant resistance to all of the agents tested, with the highest resistance levels observed for ciprofloxacin (nearly 90%), followed closely by norfloxacin (87%). In addition, the highest MIC_{50} and MIC_{90} values were also demonstrated for these agents. A total of 43.5% of isolates demonstrated resistance to all the quinolone agents tested. *Acinetobacter* strains readily develop resistance to the fluoroquinolones, a characteristic of this genus that is illustrated by the increase in ciprofloxacin resistance in the past decade. A survey of *A. baumannii* isolates collected over a 6 year period detected a decrease in susceptibility levels to ciprofloxacin from 43% in 1991 to 6% in 1997 (Rodríguez-Baño, 1999), and this appears to reflect an increase in ciprofloxacin resistance that is being reported generally (Hanberger *et al.*, 1999; Traub and Spohr, 1989; Vila *et al.*, 1993; Mokaddas *et al.*, 1998). Over half of all isolates were resistant to levofloxacin, which is in agreement with a recent survey that reported a resistance rate of 58.9% among *Acinetobacter* spp. tested (Sader *et al.*, 1998).

The newer quinolones were generally more active, although resistance rates were over 60% for all with the exception of sparfloxacin (43.6%). A recent surveillance program of antimicrobial susceptibility patterns in 6 Latin American countries reported a comparable resistance rate for trovafloxacin among *Acinetobacter* isolates (Sader *et al.*, 1998); however, a higher rate of resistance to sparfloxacin was

demonstrated by these workers (66.4%) than observed in this study. The newest generation of fluoroquinolones, which includes moxifloxacin and trovafloxacin have enhanced activity against *S. pneumoniae* and anaerobes, and were developed essentially to overcome resistance by these organisms to the earlier agents (Andriole, 1999). A study of the activity of moxifloxacin against both Gram-positive and Gram-negative organisms (including *Acinetobacter* spp.), reported that this quinolone was equal in activity to ciprofloxacin against Gram-negatives (with the exception of *P. aeruginosa*) (Peterson *et al.*, 1999), which is in contrast to this study which indicates that this particular agent is superior in activity to ciprofloxacin against multi-resistant *Acinetobacter* spp.

When comparing susceptibility rates, an important point to mention is that the majority of studies generate their resistance data using the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). However, research groups within the United Kingdom tend to use the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC) Working Party, although there appears at present to be a gradual shift towards the NCCLS guidelines in Scotland. Both guidelines employ different breakpoint values with the result that susceptibility rates of studies cannot always be readily compared, especially between countries. The resistance rates in this study were calculated using the breakpoints of the BSAC guidelines however, when these rates are altered to accommodate the NCCLS breakpoints, there is a significant decrease in the resistance rates observed for the newer quinolone agents (Table 7.1).

Using the breakpoint values of the NCCLS guidelines, approximately 52% of isolates, that were designated resistant to moxifloxacin and trovafloxacin according to the BSAC guidelines, subsequently move into the susceptible category, and 28% become susceptible to sparfloxacin.

Table 7.1 Percentage resistance rates employing BSAC and NCCLS guidelines

	% Resistance	
Antibiotic	**BSAC guidelines[1]**	**NCCLS guidelines[2]**
Ciprofloxacin	89.7	71.8
Norfloxacin	87.2	82
Grepafloxacin	64.1	51.3
Moxifloxacin	66.7	15.3[3]
Sparfloxacin	43.6	15.4
Trovafloxacin	61.5	10.2

[1] resistant = ≥ 2 mg/L (Working Party on Antibiotic Sensitivity Testing, 1991, 1998a)
[2] resistant = ≥ 4 mg/L (NCCLS, 1997)
[3] % resistance calculated using tentative breakpoint value of 1 mg/L (Andrews *et al.*, 1999)

Clearly, as these results illustrate, there is a need for the global standardisation of susceptibility testing guidelines. Although it is generally agreed that neither guidelines are perfect, a recent correspondence by Hamilton-Miller (1999) has commented that the most recent publications from the BSAC Working Party (Working Party on Antibiotic Sensitivity Testing 1998a, 1998b) more closely resemble those of the NCCLS guidelines in some respects, but as he states, "the most desirable goal of all" is " - global harmonization". Perhaps the only way this can be achieved is for both parties to accommodate the views of each other in future publications, or even better, to amalgamate aspects from both to produce one set of guidelines.

7.6.1 The mechanisms of quinolone resistance in *Acinetobacter* clinical isolates

PCR amplification of the QRDR of the *gyrA* gene of clinical isolates of *Acinetobacter* identified a substitution of Ser83 with Leu in all strains with ciprofloxacin MIC values of between 2 and 128 mg/L, whereas strains exhibiting MIC values of ≤ 1 mg/L did not produce this mutation, suggesting that this substitution is required for high level

resistance to ciprofloxacin in *Acinetobacter* spp. These findings correlate well with other studies that have also identified this change as the primary mutation in other *Acinetobacter* isolates with ciprofloxacin MIC values of ≥ 4 mg/L (Vila *et al.*, 1995; Seward and Towner, 1998). Interestingly, the latter study also detected this amino acid change in an isolate of *Acinetobacter* sp. 3, the ciprofloxacin MIC of which was only 1 mg/L, suggesting that there are other mutations or mechanisms that are involved in the development of high level resistance.

No substitutions other than at Ser-83 were identified in the present study, in contrast to Vila *et al* (1995) who demonstrated a change at Ala-84 to Pro in addition to the Ser-83 change in an isolate with a ciprofloxacin MIC value of 64 mg/L. In addition, Seward and Towner (1998) also identified a Ser-83 to Phe change in an isolate with a ciprofloxacin MIC value of 32 mg/L.

DNA gyrase is the primary target of fluoroquinolones in Gram-negative bacteria, and within GyrA of *E. coli*, mutations of Ser-83 and Asp-87, give the greatest reduction in susceptibility (Drlica and Zhao, 1997). These mutations have also been found in quinolone-resistant isolates of *P. aeruginosa* (Kureishi *et al.*, 1994), *Campylobacter jejuni* (Wang *et al.*, 1993) and *S. aureus* (Sreedharan *et al.*, 1991). In *Acinetobacter*, Asp-87 of *E. coli* is changed by Glu however, no changes affecting this amino acid were identified in this study or indeed in the studies of Vila *et al* (1995), and Seward and Towner (1998).

There is considerable conservation between the sequence of the genes encoding the A subunit of topoisomerase IV (*parC*, or *grlA* of *S. aureus*) (Kato *et al.*, 1990) and the gyrase A subunit, and it is now known that ParC is a secondary target of the fluoroquinolones in *E. coli* and other Gram-negative organisms (Khodursky *et al.*, 1995). Mutations at Ser-80 and Glu-84 of ParC in *E. coli* have been detected only in strains that already have *gyrA* mutations and at high fluoroquinolone concentrations (Chen *et al.*, 1996; Kodursky *et al.*, 1995), indicating that both *gyrA* and *parC* mutations are needed to confer higher levels of resistance. Two recent studies have

investigated whether similar mutations occur in the *parC* gene of *Acinetobacter* spp. and have identified a Ser-80 to Leu mutation in strains exhibiting high level quinolone resistance that also had *gyrA* mutations (Vila *et al.*, 1997b; Seward and Towner, 1998).

PCR analysis of the QRDR of *parC* of isolates that demonstrated a *gyrA* mutation was employed to determine whether mutations within this region were responsible for the varying MIC values observed in these strains. A Ser to Leu change at the position equivalent to codon 80 of *E. coli* was identified in the majority of isolates with ciprofloxacin MIC values of ≥ 16 mg/L, which confirmed the findings of both Vila *et al* (1997b), and Seward and Towner (1998). It has been demonstrated that compared with *E. coli*, fewer mutations are required by *Acinetobacter* in order to obtain high levels of quinolone resistance (Vila *et al.*, 1997b). The undisputed reputation of *Acinetobacter* spp. for rapidly acquiring resistance to the fluoroquinolones, as with all non-fermentative Gram-negatives, has been attributed in part to reduced uptake mechanisms that are well-known characteristics of these organisms (Amyes and Young, 1996). It is therefore possible that reduced permeability may be a contributing factor in quinolone resistance observed in these strains.

Interestingly, the *parC* mutation was not detected in 4 strains with a ciprofloxacin MIC value of 16 mg/L. Seward and Towner (1998) also reported this anomaly in an isolate exhibiting high level ciprofloxacin resistance (64 mg/L). However, in the present study, a Glu-84 to Lys change was present in the absence of the Ser-80 to Leu change in one of these isolates (830). Interestingly, this mutation has also been detected by Vila *et al* (1997b) in a high level ciprofloxacin-resistant strain which did not have the Ser-80 change, which indicates that further studies are required to investigate the role that mutations in other areas of both genes may play in quinolone resistance. Indeed, from studies involving the second sub-unit of gyrase in *E. coli* (GyrB), it is known that there are also hotspots for mutations within this gene, although it is generally considered that such mutations are rare contributors to fluoroquinolone resistance (Nakamura *et al.*, 1989). Similarily, hotspots have also

been identified in the *parE* gene of topoisomerase IV in quinolone-resistant *S. pneumoniae* (Perichon *et al.*, 1997), *E. coli* (Breines *et al.*, 1997), and *S. aureus* (Takahashi *et al.*, 1998).

Several efflux systems have been identified in both Gram-positive and Gram-negative bacteria that are known to be involved in the acquisition of resistance to several antibiotics, including the fluoroquinolones (Kaatz *et al.*, 1993; Poole *et al.*, 1993; Poole *et al.*, 1996; Köhler *et al.*, 1997). One hypothesis is that increased expression of an efflux pump is responsible for low level resistance, and may promote selection of mutations in target genes that are responsible for higher level resistance (Piddock, 1999; Pechere *et al.*, 1998). Although it is not yet clear whether similar systems are involved in fluoroquinolone resistance in *Acinetobacter* spp., it is highly likely that they are. Several isolates in the present study demonstrated variable levels of resistance to the quinolones tested, and it would be interesting to discover whether such efflux systems, or mutations other than those discussed here have an impact on the relative potencies of the newer members of the fluoroquinolone class.

7.7 The efficacy of sulbactam against imipenem-resistant *Acinetobacter* clinical isolates

Overall, sulbactam was not effective against imipenem-resistant isolates tested (MIC range 2 - >16mg/L), but lower MIC values were detected with imipenem-sensitive isolates from Scotland (0.5-2.0 mg/L). Furthermore, this inhibitor did not have a significant inhibitory effect against imipenem-resistant strains. The intrinsic activity of sulbactam against *Acinetobacter* spp. has been attributed to its affinity for PBPs 1 and 2 (Urban *et al.*, 1995), and there are several reports that have demonstrated its efficacy both alone and in combination with ampicillin against this genus (Faas *et al.*, 1996; Pandey *et al.*, 1998; Vila *et al.*, 1993; Weingarten *et al.*, 1999). The emergence of imipenem-resistant strains has prompted several workers to investigate whether sulbactam could be used effectively against them, and there is evidence to suggest that in some instances, this might be the case. Urban *et al* (1993) reported that 90% of

patients infected with imipenem-resistant strains of *Acinetobacter* improved clinically after receiving ampicillin plus sulbactam. In addition, Corbella *et al* (1998) have suggested that the use of sulbactam alone may provide an alternative therapy for infections caused by imipenem-resistant strains. However, the results of the present study suggest otherwise, and there is evidence elsewhere that supports these findings (Wood and Reboli, 1993).

Interestingly, the highest MIC value of sulbactam (4 mg/L) among the isolates from Scotland was demonstrated with the imipenem-resistant strain 6B 92 (number 792), that produces the ARI-1 carbapenemase (Paton *et al.*, 1993). To our knowledge, this strain was never exposed to either imipenem or sulbactam, but has nonetheless succeeded in acquiring a reduced susceptibility to both.

The highest MIC values were detected among imipenem-resistant isolates from Argentina where sulbactam is available for clinical use, with the result that a high percentage of strains are resistant. A recent surveillance study of nosocomial resistance in Argentina has reported a resistance rate of 65% to ampicillin/sulbactam among *Acinetobacter* spp. (C. Bantar, unpublished data).

Tazobactam demonstrated considerable less activity than sulbactam against both impenem-sensitive and imipenem-resistant isolates. Although this inhibitor has been reported to have activity against *A. baumannii* (Garcia *et al.*, 1983), there is conflicting evidence of its efficacy in the treatment of *Acinetobacter* infections (Mokaddas *et al.*, 1998; Marques *et al.*, 1997; C. Bantar, unpublished findings).

An investigation of the combined effect of imipenem and sulbactam against imipenem-resistant isolates suggested that this combination may be effective against such strains however, it was subsequently demonstrated that, although the addition of sulbactam produced a bactericidal activity with one particular strain, this inhibitor alone did not possess bactericidal activity, and its addition may in some cases antagonise the activity of imipenem.

The efficacy of imipenem and sulbactam against *Acinetobacter* is not well documented; however a lack of synergy between both agents has also been reported with an imipenem-resistant strain of *A. baumannii* (Joly-Guillou *et al.*, 1995). In contrast, Aubert and colleagues (1996) detected a superior bacteriostatic and bactericidal effect when sulbactam was combined with imipenem, although imipenem-sensitive strains were used in this study. It appears, therefore, that the combined effect of imipenem and sulbactam cannot be predicted and is very much strain-dependant.

7.8 Concluding remarks

This thesis has attempted to shed some light on the mechanisms of antibiotic resistance in *Acinetobacter* spp. and, in particular, has highlighted a growing trend of carbapenem resistance that is emerging world-wide. However, the carbapenems have been, and will remain, the drugs of choice in the treatment of nosocomial infections caused by multi-resistant *Acinetobacter*. It is therefore extremely important that the use of these antibiotics is limited as much as possible in an attempt to slow this worrying trend. The consumption of carbapenems in some areas of Argentina has increased by as much as 300% in little over a year. It is fortunate that in the UK at least, carbapenem resistance in this genus is still relatively rare. However, the discovery of the ARI-1 carbapenemase in Scotland is a reminder that this situation should not be taken for granted. In addition, the ability of *Acinetobacter* to develop carbapenem resistance via transfer of novel β-lactamases, as observed with ARI-2, also emphasises the need for alternative therapeutic choices.

The fluoroquinolones play an important role in the treatment of serious infections however, the rapid emergence of quinolone resistance in *Acinetobacter*, as observed with ciprofloxacin, indicates the range of effective antimicrobials that can be used against this genus is becoming limited. The findings of this thesis suggest that the new emerging quinolones may be effective against multi-resistant strains short-term,

but it is highly likely that the use of other quinolones may select resistance to these, with the result that their usefulness may be seriously curtailed.

Sulbactam has been an alternative agent in countries that are faced with increasing numbers of multi-resistant strains. Unfortunately, this inhibitor does not have bactericidal activity against *Acinetobacter*, and as these results have demonstrated, is ineffective against imipenem-resistant strains. Countries such as Argentina, where sulbactam is in use are now experiencing resistance rates of over 30%.

Are we now approaching the stage where antibiotics have outlived their usefulness? It appears that bacteria have always succeeded in staying at least one step ahead of man's endeavour to combat infection. Perhaps it is naïve to expect to win this battle solely with antimicrobial agents, of which so many are utilised by bacteria themselves as defence mechanisms. Research is now beginning to embrace areas other than antimicrobials, but whether these will provide a viable alternative to the current therapies available remains to be seen.

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preparations administered to individual patients by the amount of IVIG each patient received during the 5 days resulted in a mean of 15·71 mg total (active and latent) TGFβ (52% TGFβ1, 48% TGFβ2) received by the patients during the course of therapy.

We can now report that the powerful immunosuppressive cytokine TGFβ is present in substantial amounts in commercially available IVIG preparations. Since immunoglobulin has been described as a carrier of TGFβ,[4] the high concentrations of TGFβ in IVIG may initially be delivered by IVIG, namely by the immunoglobulin-bound cytokine. Because our experiments disclosed no adherence of TGFβ to IgG, the methods for the manufacture and long-term storage of IVIG may facilitate the dissociation of TGFβ non-specifically bound to IgG. The inference that TGFβ contributes to the therapeutic effects of IVIG therapy is supported by the results of various animal studies on autoimmune diseases,[5] in which the administration of TGFβ led to increased immunosuppression similar to that seen under IVIG therapy. Although TGFβ-containing IVIG preparations may be useful in the treatment of autoimmune diseases, their value in the treatment of other disorders is unknown. It remains for future clinical trials to compare the effects of IVIG preparations with and without TGFβ or to apply TGFβ alone in the treatment of human autoimmune diseases.

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Mitochondrial genotype associated with longevity

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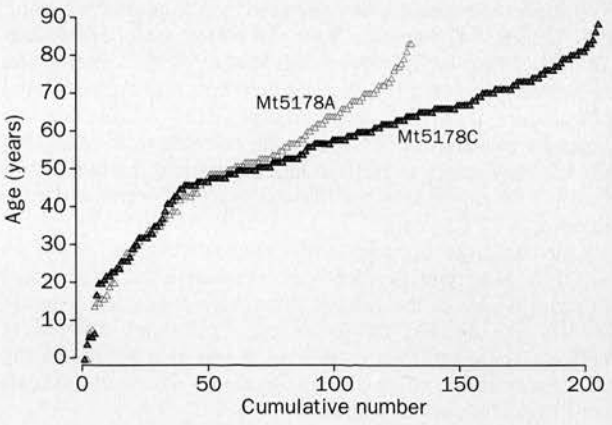
Mutations of mitochondrial DNA (mtDNA) have been reported in mitochondrial encephalomyopathies, cardiomyopathies, and diabetes mellitus. It has been proposed that accumulation of mtDNA mutations in somatic cells contributes to ageing and to degenerative diseases.[1] Sequence diversity of mtDNA is marked between individuals, because the evolutionary rate of mtDNA is 5–10 times higher than that of nuclear DNA. An epidemiological study on coronary heart disease has indicated that longevity is more strongly associated with age of maternal than age of paternal death.[2] It may be that maternally transmitted mitochondrial genotypes influence oxidative damage to mtDNA, the accumulation rate of mtDNA mutations, and susceptibility to degenerative



Age distribution of patients with Mt5178A and Mt5178C
Cumulative number of patients against age of each patient.

diseases—and thereby life span of the individual. To examine this hypothesis, we analysed mtDNA from 37 Japanese centenarians.

The entire coding regions of mtDNA, including two rRNA genes, 22 tRNA genes, and 13 protein-coding genes, from 11 centenarians were sequenced by fluorescence-based automated direct sequencing.[3] Although there were a variety of nucleotide substitutions, we identified several that were more frequent among these centenarians than among the 43 controls whose mtDNA sequences had been analysed.[4] Two nucleotide substitutions causing aminoacid replacements (a C-to-A transversion at nucleotide position [np] 5178 within the NADH dehydrogenase subunit 2 gene [*ND2*], causing Leu-to-Met replacement; and a C-to-T transition at np 8414 within the ATP synthase subunit 8 gene [*ATP8*], causing Leu-to-Phe replacement) as well as a G-to-A transition at np 3010 within the 16S rRNA gene, were more frequently observed in the centenarians than in the controls (Mt5178A in 9/11 centenarians *vs* 12/43 controls, $p<0·01$; Mt8414T in 7/11 centenarians *vs* 11/43 controls, $p<0·05$; and Mt3010A in 7/11 centenarians *vs* 11/42 controls, $p<0·05$). Most of these variations were linked; all of the seven centenarians with both Mt8414T and Mt3010A carried Mt5178A.

Among these variations, we focused on Mt5178A and screened 37 centenarians and 252 healthy blood donors in Aichi Prefecture for the presence of Mt5178A by PCR-restriction fragment length polymorphism (PCR-RFLP) with *Alu*I. The frequency of Mt5178A was significantly higher in centenarians (23/37, 62%) than in blood donors (114/252, 45% $p=0·04$, odds ratio 1·99). This finding suggests that Mt5178A is related to longevity.

To evaluate the effect of mtDNA variations on the occurrence of diseases, we analysed the frequencies of Mt5178A and Mt5178C in 338 randomly selected inpatients and outpatients at Nagoya University Hospital. The age distribution of the patients with Mt5178A or Mt5178C (figure) indicated that the frequency of Mt5178C was almost the same as that of Mt5178A among the young patients (Mt5178 A:C ratio 46:40 for patients aged <46), whereas the frequency of Mt5178C increased more markedly than that of Mt5178A among old patients (Mt5178 A:C ratio 86:166 for patients aged ≥46) with a clear reflection point at age 45. The ratio of Mt5178 A/C was significantly lower in the old patients (Mt5178 ratio 86:166) than in both the centenarians (23:14, $p=0·001$) and the healthy controls (114:138, $p=0·01$). This result suggests that the individuals with Mt5178C are more susceptible to adult-onset diseases than those with Mt5178A.

Cann *et al* reported, in their study on human evolution,[5] that among 147 samples from the world, only five Asians and one European carried Mt5178A. This observation indicates that Mt5178A is relatively rare among the global population. The high frequency of Mt5178A among the Japanese population (45%) may be relevant to the fact that the life expectancy at birth in Japan is among the highest on Earth. Life expectancy for females is 83·59 years, while for males it is 77·01 years.

Our findings support the concept that to carry an mtDNA genotype predisposing resistance to adult-onset diseases is one of the genetic factors for longevity. Further studies are needed to clarify the functional differences between these mtDNA variations. It will also be interesting to identify those adult-onset diseases to which individuals with Mt5178C are susceptible.

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Limitation of *Acinetobacter baumannii* treatment by plasmid-mediated carbapenemase ARI-2

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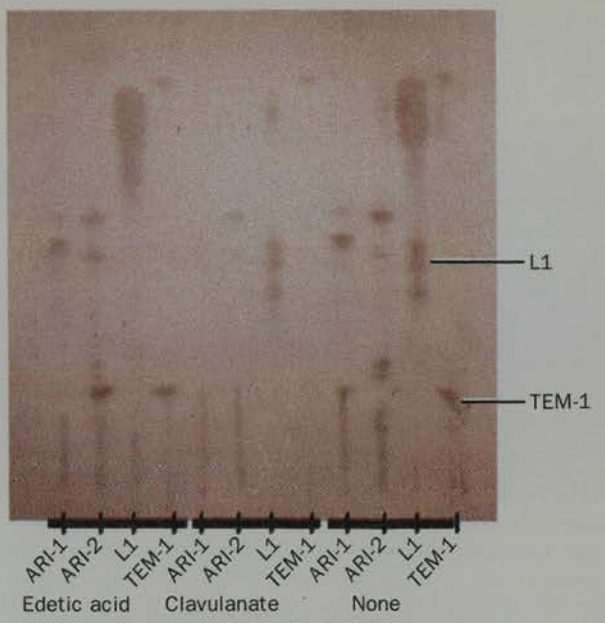
Acinetobacter species now account for 10% of nosocomial infections in intensive therapy units (ITU) in Europe. They are invariably multiresistant, and one major concern is the increasing incidence of carbapenem-resistant isolates, since these antibiotics are frequently the therapeutic choice for these infections. Carbapenem resistance in South America has already reached levels that significantly influence therapeutic choices (Carlos Bantar, unpublished data), and this looks set to be a problem in some European countries.[1] ARI-1, a plasmid-mediated enzyme capable of hydrolysing imipenem, was the first carbapenemase to be isolated from this genus.[2] We report a novel plasmid-mediated carbapenemase (ARI-2) from a multiresistant clinical isolate of *A baumannii* from Argentina (first described in 1996).[3]

The strain (1622) was isolated from a bronchial alveolar lavage specimen and identification was carried out by the API 20NE identification system. Minimum inhibitory concentrations (MIC) of a range of antibiotics were determined by the agar dilution method.[4] A cell-free extract of strain 1622 was prepared and β-lactamase bands were detected by isoelectric focusing (IEF) on a polyacrylamide gel. Inhibitors were applied to the gel to detect enzyme inhibition. Imipenem hydrolysis was demonstrated by a microbiological assay.[2] This assay was modified to increase sensitivity and quantify the activity of the enzyme.[3] Curing experiments with ethidium bromide were used to determine if imipenem resistance was plasmid-mediated. Susceptibility tests and IEF were carried out on cured colonies.



Isoelectric focusing gel showing inhibitor overlays
Edetic acid 10^{-2} mol/L, clavulanate 10^{-3} mol/L, 1=metallo-enzyme from *Stenotrophomonas maltophilia*, TEM-1=class A enzyme.

Strain 1622 was resistant to all antibiotics tested, including imipenem (MIC 16, mg/L). The addition of enzyme inhibitors, including sulbactam which is usually intrinsically active against *Acinetobacter* spp did not reduce carbapenem MIC values. IEF detected the presence of a novel β-lactamase designated ARI-2 (main band of pI 6.5). Enzyme activity was inhibited by clavulanate and BRL 42715 but not edetic acid (shown by IEF inhibitor overlays), indicating that ARI-2 was a class A β-lactamase rather than a metallo-enzyme (figure). Strain 1622 inactivated imipenem as decteted by the microbiological assay. Slow imipenem hydrolysis was calculated by the modified microbiological assay (0·75 nmol min^{-1} mL^{-1}). After plasmid curing, the ARI-2 enzyme was lost from strain 1622, detected by IEF. There was also more than a tenfold decrease in the rate of imipenem hydrolysis (0·067 nmol min^{-1} mL^{-1}), as well as a reduction in carbapenem MIC values to susceptible levels, which strongly linked ARI-2 with plasmid-encoded carbapenemase activity.

These results, and the increasing incidence of carbapenem-resistant *Acinetobacter* spp, highlight how the role of these antibiotics in the treatment of ITU infections is severely threatened. Our preliminary investigations have also demonstrated the presence of ARI-2 in a number of clinical isolates of *Acinetobacter* (including *A junii*) (unpublished), from Europe and south-east Asia. This carbapenemase looks set to become a major worldwide resistance problem which, along with the recent report of a meticillin-resistant *Staphylococcus aureus* (MRSA) isolate with reduced susceptibility to vancomycin,[5] is an indication of the alarming ability of modern pathogens to become totally resistant in response to the introduction of therapeutic strategies. The emphasis must now be on finding alternative antibiotic therapies.

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Specific HLA class I down-regulation is an early event in cervical dysplasia associated with clinical progression

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Clinical trials of a vaccine directed against HPV oncogenes for the treatment of cervical carcinoma are in progress.[1] The underlying rationale is to stimulate specific cytotoxic T-cells against high-risk HPV 16 and 18 E6 and E7 proteins whose expression is required for tumour development. The high frequency of down-regulation of HLA class I expression in tumour cells of cervical carcinomas[2] will seriously compromise the efficacy of such vaccinations because the cytotoxic T-cells generated will only recognise viral peptides in association with particular HLA class I molecules. If the loss of HLA expression in cervical neoplasia is the result of selection to avoid cellular immunity this may be an important event necessary for progression of cervical lesions during the natural history of the disease. A prospective study in which the natural history of dysplastic cervical lesions in relation to HPV status was monitored, and where no intervention occurred until the end of follow-up or when progression occurred, has provided biopsy samples to investigate this possibility.[3] The patients were recruited when cytology showed mild to severe dyskaryosis (Pap 3a/b) and were then monitored every 3–4 months by colposcopy, cytology, and HPV typing. Clinical progression was defined by an increase in CIN lesion size to more than two cervical quadrants of cytological evidence of carcinoma (Pap 5) and occurred in a period of 12 to 35 months (table).

From 88 HPV-16 positive patients of the cohort, eight showed clinical progression of their lesions. HLA class I typing of lymphocytes showed an increased frequency of HLA-B44 in the patients with lesions showing progression compared with patients with other HPV-16 positive lesions (6/8 [75·0%] *vs* 20/80 [25·0%]; p=0·0074, OR 9·0) and compared with HPV-negative patients from the same cohort (75·0% *vs* 6/30 [20·0%]; p=0·0066, OR 12·0); HLA-B44 frequency in blood donors of the Amsterdam area is 24%. This increased frequency prompted us to stratify our patients for the HLA-B44 genotype to study the HLA class I phenotype of these lesions. Immunohistochemical analysis of allelic HLA expression[2] was done on frozen biopsy sections from HLA-B44 genotype progressor (n=5) and non-progressor (n=9) patients which contained respectively histologically confirmed CIN III or various grades of CIN.

In four or five CIN IIIs from progressor patients studied there was evidence of allelic loss of HLA-B44 expression whereas no loss was seen of any HLA class I allele in the lesions from non-progressors (table). One of the HLA-B44 negative lesions showed down-regulation at all HLA-A and HLA-B alleles and another some heterogeneous expression of HLA-A29. These data are consistent with HLA-B44 class I loss occurring at an early stage of cervical neoplasia and correlate with the increased frequency of HLA-B44 in late-stage disease[4] and its frequent down-regulation in cervical carcinomas.[2]

	HLA-A				A-locus expression†	HLA-B						Follow-up (mo)	Histology biopsy (CIN)
	Genotype	Phenotype*	Genotype	Phenotype*		Genotype	Phenotype‡	Phenotype*	Genotype	Phenotype‡	Phenotype§		
Progression													
1	01	ND	03	+	+	07	+	+	44	−	−	12	III
2	03	+	29	±	+	07	+	+	44	−	−	12	III
3	02	+	24	+	+	07	+	+	44	−	−	31	III
4	02	−			−	39	−	ND	44	−	−	24	III
5	01	+	24	+	+	08	+	+	44	+	+	35	III
Non-progression													
1	02	+	24	+	+	35	+	ND	44	+	+	65	0
2	01	ND	28	+	+	39	ND	+	44	+	+	54	I
3	03	+	24	+	+	07	+	+	44	+	+	45	0
4	02	+			+	27	+	+	44	+	+	41	I
5	11	+	25	+	+	18	+	+	44	+	+	56	II
6	01	ND	23	+	+	18	+	+	44	+	+	46	II
7	02	+			+	07	+	+	44	+	+	39	II
8	02	+	25	+	ND	57	?‖	?	44	+	+	29	II
9	02	+	26	+	+	57	?	?	44	+	+	29	II

*Allele-specific monoclonal antibodies used. *GAP-A3:* A3, *H173:* A29, *H41:* A23, A24, *MA2.1:* A2, B57, *H213:* A26, *CRI1-351;* A28, *BB7.1:* B7, *MRE4.1:* B8, *H47:* B18. *ME-1:* B27, *BB7.2:* A2.

†A-locus specific monoclonal antibody (A131).

‡Monoclonal antibodies specific for Bw4 (HLA-B27, -B44, -B57) and Bw6 (HLA-B7, -B8, -B18, -B39, -B35)

§HLA-B44 specific antibody (H66).

‖These patients azre homozygous for Bw4 therefore no statement can be made about the expression of B alleles using the Bw4/6 specific antibodies. Furthermore no HLA-B57 specific antibody was available; the antibody used (MA2.1) recognises both HLA-A2 and B57.

±=Heterogeneous expression.

ND=not done or no antibody available.

HLA class I expression

These results indicate that HLA-B44 in conjunction with a down-regulated phenotype may influence rate of progression in cervical neoplasia presumably due to evasion of cellular immunity. While a tumour has potentially six different HLA class I molecules for cytotoxic T-cell restriction, the loss of expression of one allele can be sufficient to allow tumour escape from immune surveillance.[5] The results of this study may reflect the relative immunogenicity of HLA-B44 presented epitopes in HPV-associated cervical neoplasia. Loss of other HLA allelic expression (eg, specimen 4 and ref 2) as well as other factors (eg, HPV type) will also be important in contributing to the natural history of cervical neoplasia. These findings have important implications for immunotherapeutic strategies in patients with premalignant lesions of the cervix since vaccination to induce HPV-specific CTL may be ineffective at the target cell level.

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Visceral leishmaniasis in southern Croatia

Volga Punda-Polić, Sanda Sardelić, Nikola Bradarić

Visceral leishmaniasis is endemic in many parts of the world.[1] An increased incidence of the disease has been reported during the past decade in many Mediterranean countries where the disease is endemic.[2,3] Human and canine leishmaniasis has been reported in southern coastal Croatia since 1931. In this region cases of the disease had been declining and were reported only sporadically after the 1950s, probably because of mass spraying with antimalarial insecticide.[4] However, we are now concerned about an apparent increase in the incidence of visceral leishmaniasis in southern Croatia. Clinical suspicion led us to analyse records of all patients diagnosed with the infection, at the Department of Infectious Diseases, University Hospital Split, Croatia, between Jan 1, 1975, and June 30, 1997. Split is the largest city in the middle of the eastern coast of Adriatic sea, so the University Hospital Split is the regional hospital with a catchment population of about 500 000 from urban and rural areas.

A clinical diagnosis of visceral leishmaniasis was confirmed by detection of amastigotes in bone-marrow aspirate and by serology.[1] During the study period, 14 patients (six women and eight men) met the clinical and/or diagnostic criteria for visceral leishmaniasis. All 14 had fever, hepatomegaly, and splenomegaly. 11 also had pallor and weight loss. Laboratory findings were typical: raised erythrocyte sedimentation rate (nine of 14), leucopenia (nine), thrombocytopenia (eight), and anaemia (12). Plasma albumin was greatly reduced, and 13 patients had hypergammaglobulinaemia. Three cases were observed between 1975 and 1991 (two in 1983 and one in 1985). 11 (78·6%) cases were registered between April, 1992, and June, 1997, against a background of no cases in the previous 6 years. Eight of the 14 cases were among infants aged 8 months to 5 years, and six of them were under 2 years; one boy was aged 9 years. The mean age of the five adults with visceral leishmaniasis was 40·5 (SD 2·93) years. The patients infected with *Leishmania* had lived in the urban (n=4) or periurban (10) settlement of littoral (12) and in the insular area of southern Croatia (2). All our patients were previously healthy, apparently immunocompetent, and all responded to treatment with pentavalent antimonials.

The substantial increase in cases of visceral leishmaniasis was observed during the war (1992–95) and postwar period in the region. Since the disease is an opportunistic infection, the reason for the increase must be sought among changes in living conditions. Decrease of nutrition quantity and quality and a generally low standard of living during the war could have affected the immunological state of the population which would increase the risk of developing visceral leishmaniasis.[2,5] On the other hand, a change in epidemiological conditions during the war could be associated with an increase in the number of affected stray dogs, as well as disruption or irregular spraying of insecticides necessary to vector control.

In southern Croatia the disease is still a paediatric disease, since 64·3% of our patients were children under the age of 10 years. Classic visceral leishmaniasis in immunocompetent individuals in Mediterranean countries is found mainly among children, although since 1989 an increase in the number of cases among adults has been observed in southwestern European countries mostly due to *Leishmania* and HIV-1 coinfection.[2,3] For the time being, Croatia is not severely affected by the HIV-1 pandemic, but with increasing intravenous-drug use among young people in the region, the increase in HIV-1 infections is imminent. Moreover, intravenous-drug users may act as parasite reservoirs in an artificial transmission cycle of *L infantum* in the Mediterranean countries.[3]

Visceral leishmaniasis is increasing in Mediterranean countries and in southern Croatia, which suggests that we are dealing with a pattern common to the whole geographical area and emphasises the need for control and increased awareness of this parasitosis.

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An outbreak of imipenem resistance in *Acinetobacter* strains from Buenos Aires, Argentina

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Acinetobacter baumannii infections are difficult to treat because this species is often multi-resistant and so the current drug of choice is often imipenem. The ARI-1 β-lactamase, encoded by a plasmid, is the only carbapenemase found so far in this genus and has only been reported in Scotland. This unusual serine-based, presumptive class A β-lactamase hydrolyses penicillins, first generation cephalosporins and imipenem. This study reports of an outbreak of carbapenemase-mediated imipenem resistance in 13 *Acinetobacter* clinical isolates from Buenos-Aires, Argentina. Seven of the 13 isolates derived from a hospital outbreak among neonates whereas three isolates came from a separate outbreak in a different hospital. Biotyping by 20NE API strips showed 5 biotypes of *A. baumannii* and one isolate of *A. junii*. Eleven *A. baumannii* isolates and the *A. junii* strain had MICs of imipenem ranging from 8 to 32mg/l and MICs of meropenem from 4 to 8mg/l. The presence of β-lactamase and imipenem hydrolysis were demonstrated by the agar plate microbiological assay and 12 isolates, including the *A. junii* strain, displayed carbapenemase activity by this method. To detect β-lactamase inhibition, clavulanic acid was incorporated into the medium at concentrations of 2.0, 1.0 and 0.5mg/l. Inhibition occurred with 11 of the isolates, at one or more inhibitor concentrations, indicating the presence of a Class A β-lactamase. Iso-electric focusing showed that one of these strains (1622) possessed three β-lactamase bands. These focused at different positions (pI 5.8, 6.5 and 6.8) from ARI-1 (pI 6.65) and were inhibited by imipenem, clavulanic acid and BRL 42715. The results suggest that imipenem resistance in *Acinetobacter* spp. in Argentina results from novel Class A β-lactamases.

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Imipenem resistance in *Acinetobacter* from Argentina - an outbreak of carbapenemase-mediated resistance not involving ARI-1.

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Acinetobacter baumannii infections are difficult to treat because this species is often multi-resistant and, at present, the drug of choice is imipenem. Carbapenemase activity in this genus has, so far, only been reported in Scotland, and occurs as a result of an unusual serine-based presumptive Class A β-lactamase, ARI-1. This study reports an outbreak of carbapenemase-mediated imipenem resistance involving 13 *Acinetobacter* clinical isolates from Buenos Aires, Argentina which have been examined and been shown to produce carbapenemases other than ARI-1. Biotyping by API 20NE API strips showed 5 biotypes of *A. baumannii* and one isolate of *A. junii*. Twelve out of 13 strains had MICs of imipenem between 8-32mg/l and of meropenem between 4-8mg/l. Spectrophotometric analysis could not demonstrate imipenem hydrolysis by any of the isolates. Twelve isolates including the *A. junii* strain displayed carbapenemase activity by the agar plate microbiological assay. To detect β-lactamase inhibition, the serine inhibitors clavulanic acid and BRL 42715 were incorporated into the medium at concentrations of 2, 1, and 0.5mg/l, and 0.5, 0.25 and 0.12mg/l respectively. Inhibition occurred with 10 isolates for clavulanic acid at one or more inhibitor concentrations and with all 12 isolates to BRL 42715 at all inhibitor concentrations, suggesting most, if not all, are Class A serine-based β-lactamases. Iso-electric focusing showed that one of these strains (1622) possessed β-lactamase bands which focused at different positions (pIs 5.8, 6.5 and 6.8) from ARI-1 (pI 6.65). These enzymes and ARI-1 were inhibited by imipenem, clavulanic acid and BRL 42715 but not EDTA by the IEF overlay method. Induction of 1622 and ARI-1 (with imipenem at 1/4 MIC concentration) demonstrated no increase in MIC of imipenem. However, the induced 1622 demonstrated a greater enzyme activity against imipenem when tested by a modified microbiological assay method. These results suggest this outbreak of imipenem resistance is caused by novel Class A serine-β-lactamases which are not identical to ARI-1.

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ARI-2 - A plasmid-mediated carbapenemase in *Acinetobacter*?

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Acinetobacter baumannii is now recognised as an important nosocomial pathogen. Its intrinsic antibiotic resistance has led to the use of imipenem as the drug of choice. However, this therapy is now under threat with the emergence of imipenem resistant strains. A plasmid-mediated carbapenemase, ARI-1, from a clinical isolate of *A. baumannii* has, until recently, been the only reported case of β-lactamase-mediated carbapenemase resistance in this genus. This study reports of a serine-site β-lactamase which confers imipenem resistance in a clinical isolate of *A. baumannii* from Buenos Aires, Argentina. The strain (1622) had an MIC of imipenem of 16mg/l and an MIC of meropenem of 4mg/l. The presence of β-lactamase and imipenem hydrolysis was demonstrated by agar plate microbiological assay. Enzyme inhibition by clavulanic acid was detected by this method, indicating the presence of a class A β-lactamase. Two β-lactamase bands focused at different positions (pI 6.5 and 6.8) from ARI-1 (pI6.65) by isoelectric focusing. They were inhibited by imipenem, clavulanic acid and BRL 42715 overlays. A modified IEF technique showed the main β-lactamase band to be pI 6.5 (ARI-2). Plasmid curing was carried out using ethidium bromide and susceptibility testing was carried out on cured colonies. A decrease in MIC values of imipenem (0.5mg/l) and meropenem (1.0mg/l) was demonstrated in the cured strain. Isoelectric focusing demonstrated the loss of bands at pI 6.5 and 6.8 which strongly associated the ARI-2 β-lactamase with plasmid-encoded carbapenem resistance. These findings have severe implications for the future therapy of *Acinetobacter* infections.

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The differential activity of seven fluoroquinolones against multi-resistant *Acinetobacter baumannii* clinical isolates

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Acinetobacter baumannii is now universally recognised as an important nosocomial pathogen, with many outbreaks occurring in intensive care units in which extensive antibiotic use has selected for the emergence of multi-resistant strains. Many of these strains are now resistant to all commonly used antibiotics, including many of the fluoroquinolones and the carbapenems. In some countries, such as Argentina, the situation has become so bad that they are already beginnning to encounter totally resistant clinical strains of *Acinetobacter*. This study investigates the activity of seven fluoroquinolones, including the new quinolones moxofloxacin and trovafloxacin, against 41 multi-resistant *A.baumannii* clinical isolates from Argentina, Spain and Hong Kong. Fifty-six percent of these strains have previously been determined as imipenem resistant, and they represent some of the most resistant clinical Gram-negative bacteria to date. Minimum Inhibitory Concentrations (MIC) were determind by the agar dilution method, with the exception of levofloxacin which was tested by the Stokes method. At standard breakpoints, there was significant resistance amongst these strains to ciprofloxacin (68%), norfloxacin (78%) and grepafloxacin (49%). Disc sensitvity tests revealed 56% of the strains were resistant to levofloxacin. The least active antibiotic was norfloxacin with the highest MIC_{50} and MIC_{90} values (>256mg/L for both). Sparfloxacin, moxifloxacin and trovafloxacin were the most active (resistance rates of 14.6%, 14.6% and 9.7% respectively) with similar MIC_{50} and MIC_{90} values. These newer fluoroquinolones were originally developed to treat Gram-positive bacteria but these results demonstrate the possibility of a much needed role for these drugs in the treatment of severe Gram-negative infections caused by multi-resistant *Acinetobacter baumannii*.

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Transferable Spread of Imipenem Resistance in Clinical Isolates of *Acinetobacter* Isolated in Argentina, Turkey and Spain.

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The carbapenems are invariably the last choice in the treatment of *Acinetobacter baumannii* infections, however, there are now reports emerging of clinical isolates which are resistant to this class of antibiotics. We recently described a novel β-lactamase (ARI-2) from a clinical isolate of *A. baumannii* from Buenos Aires, Argentina which confers resistance to imipenem (minimum inhibitory concentration of 16mg/L). This study reports the presence of ARI-2 in other clinical *Acinetobacter* isolates from Argentina, Turkey and Spain. Eleven clinical isolates from Argentina, seven from Turkey and two from Spain were tested for carbapenem resistance by the agar dilution method. Isoelectric focusing (IEF) was used to detect the presence of β-lactamase bands in cell-free extracts. Enzyme inhibition was demonstrated by inhibitor overlays applied to the gels. Strains were differentiated by analysing DNA Restriction Fragment Length Polymorphisms (RFLPs) determined by Pulsed-Field Gel Electrophoresis (PFGE). All isolates were resistant to imipenem and meropenem with MIC values of 4.0-128mg/L for both antibiotics. IEF detected identical β-lactamase bands to those of ARI-2 in all strains from Turkey and Argentina, and in one strain from Spain. Beta-lactamase activity was inhibited by imipenem (10^{-4}M) and BRL 42715 (10^{-3}M). Partial inhibition was demonstrated by clavulanic acid (10^{-3}M). No inhibition was detected by EDTA (10^{-2}M). These inhibitor profiles were identical to that of ARI-2. PFGE revealed two distinct patterns in the strains from Argentina. A third pattern was common to all the strains from Turkey. A fourth pattern was found in the strain from Spain. These results demonstrate the presence of ARI-2 in strains of different genotype from around the world, suggesting transferable spread of the ARI-2 gene is an important factor in the dissemination of carbapenem resistance, although clonal spread may be more significant in a defined area. These findings indicate that the ARI-2 β-lactamase already plays an important role in the emergence of world-wide imipenem resistance in *Acinetobacter* spp.